AN IMMUNOLOGICAL AND OLFACTORY STUDY OF HORN FLIES, *HAEMATOBIA IRRITANS* (L.): IDENTIFICATION OF ANTIGENS FROM THE SALIVARY GLANDS AND THEIR RELATIONSHIPS TO HOST INTERACTION

BY

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

AN IMMUNOLOGICAL AND OLFACTORY STUDY OF HORN FLIES, HAEMATOBIA IRRITANS (L.): IDENTIFICATION OF ANTIGENS FROM THE SALIVARY GLANDS AND THEIR RELATIONSHIPS TO HOST INTERACTION

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Major Department: Entomology and Nematology

Studies of horn fly, Haematobia irritans, salivary antigen(s) and its response to hexane washes of repellent and susceptible cows were conducted in relation to control. Polyacrylamide gel electrophoresis (PAGE) analysis of salivary gland extract (SGE) of the horn fly showed complex proteins ranging from about 75 KDa to less than 14 KDa. Antibodies generated in rabbits against salivary gland extract (SGE) by direct immunization and that generated in mice against salivary secretions of horn flies by chronic infestation were used to immunoprecipitate protein bands from the SDS-PAGE separated and immunoblotted SGE. The mouse antisera precipitated two band at about 27 KDa. The

rabbit antisera precipitated about seven protein bands, one just below 30 KDa, two band around 21.5 KDa, three between 46 and 69 KDa and a band below 200 KDa. Antisera from both model animals detected protein bands at around 27 KDa. The antigen at 27 KDa was therefore identified as a secretory protein that can have immunological importance. Use of the two types of polyclonal antibodies generated to probe histological sections of the head and thorax of the horn fly showed that the rabbit antisera specifically localized the epithelial cells making up the salivary glands. The mouse antisera produced nonspecific binding when compared to normal mouse serum.

Periodate oxidation test showed that the SGE possesses a carbohydrate epitope. The optical density of periodate treated SGE was reduced by a greater percentage when reacted with polyclonal antibodies from the rabbit than from the mouse.

Use of the ten-port olfactometer for testing hexane washes of susceptible and attractant bovines and selected natural chemicals supplied by International flavors and fragrances (IFF) showed the olfactometer to be a reliable tool for testing efficacy of chemicals against horn flies. Also, the result showed that the individual host animal phenomenon of resistance and attractancy to horn flies is real suggesting further research to identify the chemicals produced by the host which produces the repellent activity.

CHAPTER 1
LITERATURE REVIEW AND RESEARCH OBJECTIVES

Importance Of The Horn Fly

About half of the annual agricultural income in the United States of America comes from livestock (Kunz et al., 1991). USA livestock farming is considered one of the most efficient and productive in the world, partly because of modern methods used to control parasites of these animals. Despite great success, there remains a need to control some ectoparasites and replace failing methods to prevent further losses and to provide for more efficient production. Most discussion of ectoparasites dwells on their role as vectors of disease, but the direct costs of hematophagous ectoparasites are of great economic importance. The horn fly, Haematobia irritans (L.), a muscoid fly of Old World origin, is considered one of the most important ectoparasites that adversely affects cattle production. This species arrived from Europe between 1884 and 1886 (Riley, 1889; Marlatt, 1910; Dorsey et al., 1962; Bruce, 1964; Butler, 1990). Both sexes of the fly are obligate, blood-sucking ectoparasites of cattle but will also feed on other mammals. Adults feed continuously, taking about 24-38

blood meals a day (Harris et al., 1974). Horn flies spend their immature stages in the host's manure. Only the adult stage is parasitic and hematophagous.

Horn fly populations parasitizing cattle in Europe are relatively low, averaging about 200 flies per animal or fewer (Hammer, 1942). Extreme numbers, however, have been observed in the tropics and semitropics, ranging from 1100 per animal for beef cattle to as many as 20,000 for bulls (McLintock and Depner, 1954). In the USA significant economic damage to cattle occurs when horn fly density exceeds 50 flies per animal (Butler, 1975). In contrast, Schreiber et al. (1987) and Hogsette et al. (1991) reported the economic injury level to be about 200 flies per animal. Damage caused by the horn fly includes reduction in normal weight gain and milk production. Cost to the cattle industry of horn fly infestation in the USA is about $876 million per year (Kunz et al., 1991).

Since the mid-1940's control of horn flies on cattle has been based primarily on insecticides. These methods are effective because the horn fly remains on the host and leaves the host only to deposit eggs. But the continuous use of insecticides has resulted in the development of resistance to many insecticides (Sheppard, 1983, 1984; Byford et al., 1985; Kunz and Schmidt, 1985; Schmidt et al., 1985; Cilek and Knapp, 1986; Steelman et al., 1991). Resistance to insecticides developed rapidly, and efforts

have been made to prolong the efficacy of available pesticides (Cilek and Knapp, 1991). Because of concerns of resistance, multiple resistance, cross-resistance to the major classes of pesticides, ecological awareness and escalating prices of petroleum-based pesticides, alternative control measures are receiving greater emphasis, especially integrated procedures that include anti-arthropod vaccines. Anti-arthropod vaccine have been developed for tick control. It works by conferring on the host the capacity to inhibit parasite attack (Wikel and Allen, 1982; Agbede and Kemp, 1986). Similar work by Schlein and Lewis (1976) on hematophagous flies shows increased mortality and cuticular abnormalities when flies feed on rabbits immunized with crude extracts of fly integuments and other tissues.

In investigating alternative control techniques, it is essential to understand the interaction between host and parasite. Information on the immunological aspect of the interaction could form the basis for the development of an anti-arthropod vaccine. It may also provide an understanding of the genetic basis of natural resistance to parasites. This could lead to the development of livestock breeds with enhanced genetically based resistance to arthropods.

Evidence from cattle studies demonstrated that breed and color influence horn fly infestation, and individual cows within the same breed show different susceptibilities

to horn flies (Frank et al., 1964; Tugwell et al., 1969; Holrroyd et al., 1984; Brethour et al., 1987; Cocke et al., 1989; Steelman et al., 1991 and Steelman et al., 1993). Low pest populations on individual cows also have been attributed to dislodging or repelling behavior of hosts as fly biting density increases (Harris et al., 1987). The phenomenon of refractoriness or attractiveness of individual cows within several breeds to ticks has been reported (Riek, 1962; Wilkinson, 1962; Wharton et al., 1969; Seifert, 1971; Latif, 1984; Latif et al., 1991) and selective breeding has been used to enhance resistance of host animals to ticks and lice (Riek, 1962, Clifford et al., 1967, Wharton, 1974, Sutherst et al., 1979).

Although research has been carried out on the life cycle and basic biology of the horn flies, little has been done on the immune response they elicit in hosts. Kerlin and Allingham (1992) demonstrated that cows exposed to buffalo flies, Haematobia irritans exigua, developed antibodies to buffalo fly antigens at levels correlated to intensity of exposure, but flies fed on animals with high antibodies in their serum did not show greater mortality than flies fed on unexposed animals. Development of anti-arthropod vaccine would require the identification of specific antigens that can confer immunity.

Investigation of the immunological relationship between the horn fly and its host and of factors influencing

differential infestations of cows by horn flies could provide information useful in the development of control measures that could be used in an integrated scheme.

Control Strategies For The Horn fly

Horn fly attack on cattle causes pain and annoyance that interferes with feeding and resting. Horn fly feeding cause laceration, which results in open sores on the head and under-body of cattle which may lead to secondary infection caused by pathogens and parasites such as the screwworm, Cochliomyia hominivorax (Greenberg, 1971). The piercing-sucking mouthparts (Fig. 1.1) enable the flies to transmit anaplasmosis and other diseases mechanically within the herd (Greenberg, 1971).

The importance of methods for horn fly control on cattle cannot be underestimated in this era of strict regulation of allowable chemical in food. Control of horn flies presents unique problems because the immature stages develop in fresh manure and only the adult stages are parasitic on the host. The adults' habit of staying on the host makes it susceptible to chemical control methods. Most chemical control is directed at the adult or against the immature stages. Studies show that cattle treated with insecticides gain weight more rapidly than untreated animals (Duren and O'Keefe, 1972; Campbell, 1976; Haufe, 1982; Kinzer et al., 1984 and Quisenberry and Strohbehn, 1984).

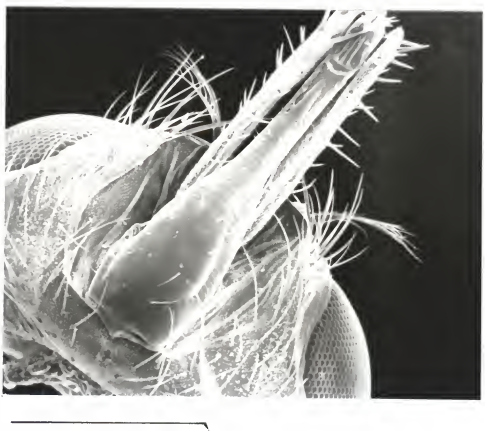

Figure 1.1. Scanning electromicrograph of the mouthparts of the horn fly. Scale bar = 0.2 mm.

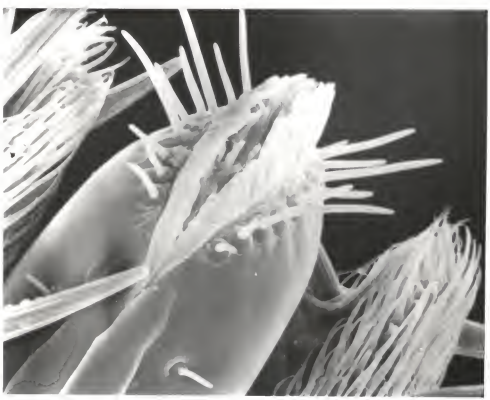

Figure 1.2. Scanning electromicrograph of the labium of the horn fly mouthpart showing the prestomal teeth. Scale bar = 0.2 mm.

Control of horn flies throughout the USA relies heavily on chemicals. Beginning in the mid 1940s' DDT and other organochlorines and arsenic chemicals were registered for horn fly control (Laake, 1946; Matthysse, 1946). Although resistance to DDT and methoxychlor was later confirmed (Harris,1964), a variety of chemicals still existed for horn fly control between 1960 and 1965. In addition to the organochlorines, organophosphorous compounds such as fenchlorphos^{R}, crufonate^{R}, malathion^{R} and carbamates like carbaryl^{R} were available. Resistance of flies to toxaphene and fenchlorphos soon developed. Introduction of insecticide impregnated eartags initially provided economic and effective season-long control (Ahrens, 1977; Ahrens and Cocke, 1979; Sheppard, 1980;). Unfortunately, resistance soon developed to the organophosphorus insecticide used in the first ear tags (Sheppard, 1983). Stirofos^{R} impregnated ear tags were then replaced by those containing one of the pyrethroids, permethrin, or fenvalerate. In the USA, control problems with the pyrethroids were reported when the tags had been in use for 2 to 4 years in the southeastern USA (Quisenberry, 1984). Despite problems, insecticide impregnated ear tags remain the formulation of choice, because they fit well in herd management systems and provide up to 6 months' control of horn flies (Ahrens and Cocke, 1979). The resistance picture of pyrethroid ear tag has been summarized by Butler (1992) (Fig. 1.3).

Currently, one of the most serious problems facing agriculture globally is the development of resistance to most registered chemicals. This often results from the overuse or misuse of the chemicals. Horn fly resistance is attributed in part to the elution rate from ear tags, which diminishes over time (Miller et al., 1984), leading to exposure of flies to sub-lethal dosages of pesticides. The loss of pesticide efficacy for horn fly control has made control difficult and costly, creating problems for both cattle producers and entomologists because of the limited supply of replacement pesticides and the tremendous cost for development of new pesticides (Georghiou, 1986). The cost of developing new pesticides is now more than the economic return before development of resistance. Few pesticides are therefore being developed.

Biological control methods targeted at the immature stages of the horn fly had some impact on controlling the horn fly and its sub-species the buffalo fly, Haematobia irritans exigua (Degeer), from Australia. Worldwide investigations of natural enemies of the horn fly dealt with four categories: (1) predators (2) parasites and parasitoids, (3) pathogens, and (4) competitors. Introduction of dung-burying scarab beetles from Africa to augment those in Australia and the United States in the 1970s did not reduce horn fly or buffalo fly populations. This failure was attributed to the horn fly's greater

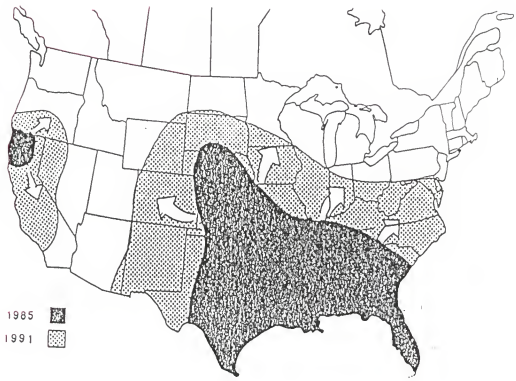

Fig. 1.3. Probable distribution of horn fly resistance to pyrethroid ear tags (Butler, 1992)

ability to disperse to fresh dung compared to the scarabs. Sufficient horn fly immatures managed to complete development in dung before scarabs buried them. Climatic differences between the native African conditions and the Australian and American conditions also resulted in scarabs being unable to build up high early season population levels in their new environments. Burying of the dung also interfered with the effect of native predators on horn flies (Macqueen, 1975; Legner, 1978; Roth et al., 1983), and horn fly populations increased rather than decreased (Harris, 1981).

Use of the sterile technique as a component in an integrated control program was tried for the horn fly in Brazos County, Texas. Control could not be achieved because the target area could not be isolated (Kunz et al., 1974). Eschele et al. (1977) reported the elimination of horn flies from semi-isolated areas for two weeks by using a combination of orally administered methroprene insecticide treatment along with sterile male releases on the island of Molokai in Hawaii. The feasibility of using the sterile insect technique for large scale horn fly control is not known presently.

Possible horn fly control strategies that could be used in an integrated control program include enhancing resistance immunologically through immunization, or developing livestock breeds with genetically based

resistance to arthropods. Much work in this area has been done with ticks. Trager (1939) was a pioneer researcher in investigating the possible use of host immune response to control arthropods. Materials from the salivary glands and various other internal tissues of ticks were found to be responsible for inducing tick resistance to animals (Manohar and Banerjee, 1992). Application of some of the methodology in the work with ticks could unveil information on horn fly antigens that might induce acquired immunity in cows. Also, understanding of the host-parasite relationships could enable the selection and breeding of cows that are naturally resistant to horn fly infestation.

Host-Parasite Interaction

Man and animals have been plagued throughout the centuries by arthropods that consume enormous quantities of food and are responsible for numerous diseases transmitted either by them or caused by the insects themselves (Benjamini and Feingold, 1969). Of these, the blood-feeders are of great importance because they vector some of the most dangerous parasites. Little information exists about the early interaction between parasites and their mammalian hosts, but it is documented that parasites, together with their hosts, underwent significant evolutionary radiation. It is believed that parasitic arthropods developed through a series of transitional stages from free-living forms to

obligatory parasites. The existing relationships between parasitic arthropods and mammals are a result of these interactions during the past thousands of years (Kim, 1985).

Nelson et al. (1975) described ectoparasites as semi-independent organisms living on host surface but with the ability to live free from their hosts for short periods or to move from one host to another. They were divided into 3 groups according to habits or ecological niches: (1) those feeding only for a limited period on their host and free-living for most of their life cycle (e.g., Ixodidae: Acarina); (2) nest ectoparasites most often collected in the habitat of their host rather than from the host itself (e.g., Argasidae: Acarina and Siphonaptera) and (3) host ectoparasites that are permanent residents of the host's integument (e.g., Anoplura and Mallophaga).

Wakelin (1984) identified nutrient transfer and energy exchange as central in the host-parasite relationship. Insect parasites exhibit high levels of nutritional, physiological and behavioral interactions with their host, with most ectoparasites feeding on host fluid or integument. In the host-parasite system, parasites exploit the host, and, in response, the host reacts to minimize that exploitation. Blood feeders, like mosquitoes and ticks, cause the most damage often causing anemia. Blood loss is correlated with feeding habits of many ectoparasites (Nelson et al., 1977).

As ectoparasites feed, they also inject their salivary secretions into the host, triggering host defense responses. The responses provide new information, and the parasite make counter-responses. This sequence of responses and counter responses results in adaptations in behavior, physiology, development, and even morphology for both the parasite and host (Whitfield, 1979). Host response to ectoparasitic attack can be (1) a localized traumatic reaction to injury caused by the insects' mouthparts, (2) a toxin injected into the wound in the insect's saliva, or (3) an immune response to an antigen in the saliva (Lehane, 1991). The response were summarized as immunologic, allergic, traumatic, toxic or irritant by Nelson et al. (1977). Physiological consequences of hematophagy on vertebrate hosts, as reviewed by Nelson (1989), include metabolic changes, anorexia, inflammation and immune sensitization, all of which interact to reduce productivity. Stable fly, Stomoxys calcitrans (L.), and horn fly, Haematobia irritans attack, cause changes in heart rate and respiration, nitrogen balance and body temperature of the host (Schwinghammer et al., 1986a, b).

Lower susceptibility to ectoparasitic, hematophagous arthropods may be acquired through the host's immune system (Wikel, 1982). Though hosts' respond immunologically to arthropod feeding, only in few cases are these responses associated with development of acquired resistance (Baron

and Nelson, 1985). Host defenses against parasites occur at both behavioral and physiological levels and the sum of all the physiological potential of a host that protects it from parasite infestation is called resistance (Kim, 1985). Mammalian hosts exhibit both natural and acquired immunity to arthropod infestation. Levels of acquired resistance likely reflect a dynamic balance between the immune rejection mechanisms of the host and the immunosuppressive capabilities of the blood feeding arthropod (Ramachandra and Wikel, 1992). Immunity can be developed to parasitic and predatory arthropods or arthropod fragments (Benjamini and Feingold, 1969). Acquired immunity to arthropods may be roughly grouped into three major categories: (1) immunity associated with the neutralization of toxic substances introduced into the body by arthropods (eg., venoms of scorpions, spiders, bees and wasps), (2) hypersensitivity to antigens of arthropod origin (eg. reactions to arthropod bites, stings, urticating hairs and spines, allergy induced by inhaled arthropod fragments, and allergy induced by ingested arthropods), and (3) immunity to infestation or invasion by arthropods (eg. infestation by certain ticks and invasion by certain myiasis-producing dipterous larvae). This division is of practical value, but arbitrary since these categories are not necessarily mutually exclusive.

Most arthropods introduce antigens into hosts through mouthparts. This makes hematophagous arthropods

parasitizing vertebrates the best candidates for investigation of host immune response (Benjamini and Feingold, 1969). Apart from disease transmission, the manner in which hematophagous arthropods obtain their blood meal often triggers a hypersensitive reaction by the host. This means that the host responds immunologically to the arthropod or arthropod products. This reaction may be unpleasant and could lead to severe manifestations ranging from pruritus and generalized urticaria to systemic anaphylaxis and death. The host's reaction to the bite of the hematophagous arthropod has been studied for many arthropods, both from the stand-point of the host-parasite relationship and from the standpoint of its possible role in disease transmission. By virtue of dependence on the host for survival, the degree of reactivity of the host to the bite of the arthropod may play a role in the host-parasite interrelationship. This may also affect infectivity with pathogens and their survival in the host. Host hypersensitivity to salivary antigens of arthropods may cause severe reactions to bites of a normally mildly biting arthropod such as the Anopheles mosquito. Trauma caused by arthropods that lacerate the skin with their blade-like mandibles (e.g., deer flies, black flies, stable flies, and tsetse flies) is more severe and noticeable by the host than that caused by those possessing piercing mouth parts (e.g., mosquitoes and fleas) (Benjamini and Feingold, 1969).

Nelson et al. (1977) reported that antigen (oral secretion) deposited in skin tissue stimulates host's immune system via the lymphatic route, while those injected intravascularly probably make their first contact with antibody-forming cells. He also reported that in many biting flies (e.g., mosquito or horn fly) and ticks both the lymphatic and the circulatory route are involved. Cows exposed to the buffalo fly (Haematobia irritans exigua) have been documented to develop serum antibodies to buffalo fly antigens that correlated with the intensity of exposure (Kerlin and Allingham, 1992).

Innate host resistance is heritable and includes the hosts' ability to groom itself, as well as encompassing inherited factors like skin color and odor. Acquired resistance describes the ability of the host to interfere with fly feeding, due to previous exposure or immunization.

The interaction of antibodies with the horn fly is little understood and the identification of the antigen(s) that elicit humoral responses in the host cows has not been undertaken.

Arthropod Defence Mechanism

In nature, ecological stability exists between parasitic arthropods and their hosts. Insect immune system developed to protect them from potentially damaging effects of biological invaders (Lehane, 1991). The ability to

protect themselves by removing microorganisms and parasites from their circulation has contributed greatly to the success of insects (Azambuja et al., 1991). Immune strategies also modulate the effects of host immunologic effector mechanisms induced by insect parasitism. This allows the parasite to survive in the face of host antibodies (Leid et al., 1987). A potent humoral immune system has been induced by injection of live non-pathogenic bacteria in insects (Gotz and Boman, 1985). Factors governing immune reaction are located in the hemolymph in both cellular and humoral immunity. The insect immune system is not homologous to vertebrate systems in distinguishing foreign entities. Using immune reactions of vertebrates as evaluative criteria would make insects and other invertebrates immunocompetent (Good and Pappermaster, 1964; Saunders, 1970). Two major features of vertebrate immune response lacking in the insect immune response are specificity (antigen-antibody complementarity), and increased responsiveness to foreign substances as a result of prior exposure (immunologic memory). Nevertheless, insects are capable of effectively discerning and combating foreign antigens (Nappi, 1975). Some insects produce relatively nonspecific substances (antimicrobial, antibacterial, lytic) against various invading microbial organisms that afford the host some defence (Whitcomb et al., 1974).

Cellular immune mechanisms of insects are mediated by the cellular components of the insect hemolymph (hemocytes) that eliminate invading organisms by phagocytosis and/or encapsulation. Encapsulation defines a coordinated response involving the aggregation, adhesion, and flattening of hemocytes over foreign surfaces too large to be engulfed by individual cells. Generally encapsulation reactions in insects are accompanied by the intra and extracellular deposition of melanin pigment (Whitcomb et al., 1974). Most vertebrate parasites that use insects as biological vectors are directly or indirectly affected by the hemocoel of the vectors (Weathersby, 1975).

The immune response in insects is acquired and is an individual characteristic. It is of little practical use to control vectors. Many vertebrate parasites that might be expected to produce acquired immunity in insects either do not penetrate to the hemocoel or they cause very high mortality in the vectors. Often, the vector only lives long enough to transmit the causal agents of diseases and there is little opportunity to determine immunity. Some insects may acquire the ability to transmit a parasite to offspring but it is not likely that an antigenic stimulus will affect the organism genetically. Although insects produce specific substances in response to foreign substances and antigenic stimulation, they appear to have little resistance to overcoming many parasites (Huff, 1940).

The description of the arthropod immune system as primitive and unsophisticated, compared with that of vertebrates, is becoming untenable. At least six monosaccharides or their derivatives found on the plasma membrane of vertebrate cells are present on the immunocyte plasma membrane of one of the most primitive arthropods, the horseshoe crab Limulus polyphemus (Gupta, 1991). They most likely participate in foreign tissue recognition, phagocytosis, and encapsulation, and at least one (sialic acid or NANA) is involved in signaling and cell-cell recognitions and activation of the complement pathway (Gupta, 1991). Furthermore, specific inhibitors of the classical and alternative complement (C) pathway of mammalian complement are present in the insect hemolymph. It is now thought that the key component of the vertebrate complement exists in insects. Evidence is accumulating that in some insects, a humoral adaptive response that possesses specificity and memory comparable to the functional attributes of the mammalian immunoglobulins exists.

Extensive studies have been done on humoral immunity in lepidopterans, but relatively little has been done on dipterans (Kaaya et al., 1987).

Vertebrate Immune System And Host-Immune Response.

The immune system of vertebrates includes bone marrow, thymus, bursa of fabricius, spleen, lymph nodes, gut-

associated lymphoid (GALT), and the reticuloendothelial system (Clark, 1986). This system performs the task of protecting the host from threats to homeostasis, both exogenous and endogenous (Bigley et al., 1981). A coordinated system of cellular communications enables host cells to differentiate between "self" and "non-self", and react appropriately to eliminate foreign antigens (Nappi, 1975). Depending on the nature of the antigen, one of two major forms of immunologic effector mechanisms may predominate in destroying or eliminating the antigenic material. A substance which when introduced into an organism induces an immune response is called an antigen. Its immunogenicity refers to its capacity to stimulate an immune response under a set of conditions. Severity and type of antibody response in hosts depends on antigen chemical structure, physical state, stability, size, frequency of presentation, route of presentation (oral, dermal, respiratory etc.), complexity of the array of immunogens, and presence of other factors (e.g presence of adjuvant that allow antigens to remain at site) and idiosyncrasies of the host (Spiegelberg, 1974). Response to potential antigens varies from no response e.g. high-zone or low-zone tolerance (Clark, 1986) to severe allergic hypersensitive reactions. Once detected, invading antigens are identified, and processes for their elimination are initiated.

The vertebrate immune response is made of two systems: the humoral and cellular systems (Clark, 1986). In the humoral system a specific B-cell recognizes and interacts with an immunogen, it is then activated to differentiate into a plasma cell which then undergoes multiplication and forms clones of the original plasma cell. The resulting plasma cells synthesize and secrete specific glycoprotein molecules (the antibody or immunoglobin) into the blood plasma. Some antigens stimulate the thymus gland, and this results in the appearance of "T" lymphocytes, with effector activities such as the ability to kill cells by membrane contact or to elaborate soluble products that assist in the development of inflammatory responses. In the cellular response, a "T" cell recognizes and binds to the antigen leading to the ultimate elimination of the antigen from the system (Clark, 1986). Mammals also utilize immediate and delayed mechanisms for protection (Raven and Johnson, 1987). The immediate mechanism functions by first isolating the entrance site through the inflammation process (Wakelin, 1984). The immune response then attempts to destroy the invading organism by phagocytosis, utilizing macrophages and granulocytes such as neutrophils, eosinophils, and basophils. The delayed system, consisting of a humoral or B-cell system and a cell mediated or T-cell system, is turned on by the above phagocytosis, forming antibodies and sensitized lymphocytes.

Host response to arthropod bites varies among species, among individuals of one host species, and also with arthropods doing the biting (Nelson et al., 1977). A general sequence of skin reactivity has been noted in time in hosts repeatedly exposed to insect bite and has been broadly divided into five phases. These phases of the sequence were described from studies of rabbit and human responses to mosquito bites (Mellanby, 1946; McKiel and West, 1961) and to guinea pig responses to flea bites (Benjamini et al., 1960). Feingold et al. (1968) described the phases as follows:

Phase I. Induction. No response or reaction, but foreign material is identified by Langerhan's cells and macrophages as antigenic.

Phase II. Delayed hypersensitivity reaction. Dermal responses to antigen appear 24-48 h after exposure and include redness, swelling, and itching at site of exposure (Dahl, 1987). Delay is caused by time required for specific leukocytes to migrate to site of exposure and release lymphokines that recruit other leukocytes and cause capillaries to vasodilate, resulting in edema and infiltration of erythrocytes.

Phase III. Delayed and Immediate hypersensitivity reaction. Delayed and immediate reactions occur jointly, with immediate reactions causing redness and swelling within 15 minutes (Clark, 1983; Dahl, 1987). Immediate responses are

rapid because plasma cells elaborating immunoglobulin class E (IgE) are present. The IgE binds to Fc receptors of mast cells in the dermis and are available for immediate binding with antigen.

Phase IV. Immediate hypersensitivity reaction. Delayed responses have waned, perhaps because of deletion of T-helper cells (Dahl, 1987), or more likely because T-suppressor cells have appeared and are suppressing the delayed response (Dahl, 1987, 1989; Van Neste, 1988).

Phase V. Desensitization or Hyposensitization or no reaction. No hypersensitivity occurs on exposure to antigen perhaps because of the effect of T-suppressor cells (Dahl, 1987, 1989) or because IgG is blocking the IgE-mediated reaction by direct competition for antigen (Dahl, 1989). Here, antibody-producing cells have been exhausted or are suppressed. These groupings are not mutually exclusive.

The development of acquired host immunity to arthropods as a means of ectoparasite control is of scientific interest (Preutt and Thomas, 1985). Research by Wikel (1982) suggests that hosts' lower susceptibility to ectoparasitic, hematophagous arthropods may be acquired through the host immune system. Results of immunoglobulin participation in the development of acquired immunity could be associated with host skin reactions that disrupt feeding efficiency or react with target antigens within the gut or hemocoel of the arthropod interfering with digestion or metabolism. Primary

targets might include salivary gland secretions, intestinal symbionts, digestive enzymes or secretions of the gut such as digestive enzymes or the peritrophic membrane (Pruett and Thomas, 1985).

Host Location By Arthropods

The survival of parasitic species depends on their selection or location of suitable hosts. Host location is a difficult and complex behavioral task involving an integrated but flexible behavior package which gathers momentum as a host is tracked down (Lehane, 1991). Species that actively search for hosts move towards stimuli, while passive species wait either on vegetation or within refuge sites until a host comes within reach (Waladde and Rice, 1982). The sequence of behaviors involved in host searching are susceptible to manipulation or interference by humans (Colvin and Gibson, 1992). These behaviors though do not occur in a strict and inflexible sequence (Lehane, 1991). The effectiveness of chemical control of adult flies is based on the knowledge of their host seeking and resting behavior. Generally only exogenous factors can be manipulated in field populations. Host seeking and location in insects is guided by both chemical and physical cues originating from the environment and is elicited by visual, thermal, humidity, and chemical stimuli operating separately or in combination (Laarman, 1955; Dethier, 1957; Brown,

1966; Gillies and Wilkes, 1969; Hocking, 1971; Vale, 1977; Dalton et al., 1978; Kinzer et al., 1978). Some nonchemical factors influencing host selection and location include wavelength and intensity of light reflected from the host.

Two major sensory inputs of recognized importance are olfaction and vision. The importance of vision in host location has been demonstrated (Parr, 1962; Brady, 1972; Vale, 1974). The response of blood-sucking flies to carbon dioxide and host odors has been studied in the field (Vale, 1980) and in the laboratory (Gatehouse and Lewis, 1973; Mayer and James, 1969). Host odor (Hargrove and Vale, 1978) and the odors of excretory products (Owaga, 1984; 1985) have been found to be highly attractive to tsetse (Glossina spp.). Identification of components of such odors has led to the development of effective baits for sampling or controlling tsetse populations (Vale et al., 1986). The chemical, 1-Octen-3-ol, was isolated from cattle odors and has been found to be a potent olfactory stimulant and attractant for tsetse (Hall et al., 1984). Krijgman (1930) conducted experiments with the stable fly, Stomoxys calcitrans, in a simple olfactometer in still air and reported orientation to the odor of fresh horse blood. The same species failed to respond to olfactory stimuli from blood when the odor was dispersed by moving air (Gatehouse and Lewis, 1973). Tests of various components and fractions of blood as attractants resulted in the discovery of an

extremely volatile constituent which attracts Culex mosquitoes and Stomoxys. It appears that the material acts as an attractant to which the insects orient, and as a releaser for the act of piercing. It is believed that this volatile fraction of blood diffuses through the skin of the host and is an important factor in attracting mosquitoes and biting flies to the host (Galun, 1975a). Attractants can be either close range or distant depending upon how far away stimulation of olfactory response occurs from the source. It is uncertain how far odor signals travel before they fail to be detected. In nature, horn flies show preference for attacking some cow breeds and color, whereas, others are not attacked even though they are close to those attacked. Upon eclosion, horn flies depend more on vision than olfactory or host stimuli to locate their hosts (Hargett, 1962; Hargett and Goulding, 1962; Milstrey, 1983). Horn flies are influenced by light stimuli and negative geotaxis, and fly up into the air. Kinzer et al. (1978) observed that temperature and Co_2 were prime factors in horn fly orientation. Dalton et al. (1978) found radiated rather than internal heat and host color to be the most important factors influencing flies, especially in close quarters. Milstrey (1983) observed that horn fly population and migration was regulated in part by semiochemicals. Bolton (1980) and Mackley et al. (1981) showed that in horn flies 4 olefins, (Z)-9-tricocene (masculure), (Z)-5-tricocene, (Z)-

9-pentacocene and (Z)-9-heptacocene serve as mating and aggregation pheromones. Milstrey (1983) showed that blends composed of equal parts of all 4 olefins and a combination of (Z)-9-heptacosene had little effect. An olefin blend (50:40:6:4) of (Z)-tricocene, (Z)-5-tricocene, (Z)-9-pentacocene and (Z)-9-heptacocene best reflected natural pheromone levels in the field. This blend significantly reduced horn fly numbers on cattle and attracted them on horses for a short period of time. Horn flies are thought to lay pheromone trails which in higher concentrations causes flies to migrate. Chamberlain (1981) showed that horn flies locate their host by attraction not by passive encounter.

Hosts possess characteristics which influences parasite infestation, and these have been exploited for some pest control. Detection of odors by insects is presumed to result from changes in the electrical activity of primary olfactory receptor neurones contained within the antennal sensilla (Grant and O'Connell, 1986). The majority of studies on odor-mediated flight orientation in insects has involved upward flight of male moths toward a source of female pheromones as a model (Kennedy, 1986). Almost all of these observations have been made in laboratory wind tunnels. Attractive compounds that have been identified in cattle odor for tsetse flies include carbon dioxide, acetone and 1-octen-3-ol (Bursell, 1984; Hall et al., 1984; Vale

and Hall, 1985). The screening of compounds for their olfactory effectiveness by fly trapping in the field is time consuming and subject to many variable factors. Olfactory components are responsible for these effects. The components can be exploited for control purposes.

The complex behavioral repertoire culminating in host-location by hematophagous arthropods involves an array of chemical and physical cues (Chapman, 1961; Hocking, 1971; Friend and Smith, 1977). It is now well documented that carbon dioxide emanating from the host constitutes a primary chemoattractant to blood sucking ectoparasites (Garcia, 1962, 1965; Wilson et al., 1972).

Role Of Saliva In Blood-Feeding By Arthropods

In their quest for a blood meal, hematophagous arthropods introduce an array of salivary compounds into the host. The saliva stimulates host immune response alerting the host to the presence of an arthropod. The immune response ultimately may deny a blood meal to the arthropod. Salivation may look detrimental to an insect, but studies have identified some benefits to the insect (Lehane, 1991). The function of saliva in many important insect vectors are poorly understood (Kerlin and Hughes, 1992). These authors reported that probable salivary secretions from parasites promote feeding and survival on the host. Many functions have been attributed to the saliva of blood-feeding

arthropods. The first array of digestive enzymes is found in the salivary glands. Among other things, the contents of the saliva facilitate blood feeding by blocking the hemostatic reactions or by causing allergy hemodynamics. Saliva from Ixodes damini have been found to contains anti-hemostatic, immunosuppressive, anti-inflammatory, and neutrophil inhibiting components (Ribeiro et al., 1985). Anticoagulants which prevents coagulation of blood which is capable of blocking the mouthparts of the insect have been found in saliva or salivary gland homogenates of certain hematophagous arthropods but not in others (Gooding, 1972). Also hemagglutinins have been found in the salivary glands of some hematophagous arthropods but their roles remain unknown (Gooding, 1972).

Most pathogens transmitted by ticks are introduced into host bodies with saliva (Hoogstraal, 1970; Binnington and Kemp, 1980). Tick salivary gland secretory products include: (1) cement to help anchor the mouth parts, (2) anticoagulants in some species, and (3) antiinflammatory and immunosuppressive molecules that assist in feeding and evading host defense mechanisms (Binnington and Kemp, 1980; Ribeiro et al., 1985). Host immunity can be elicited in response to antigens secreted by the salivary glands. The size, mass, and protein content of the salivary gland increase approximately 25-fold during tick feeding (McSwain et al., 1982).

It has been recognized that for any blood-feeding ectoparasite, the range of antigens presented to the host in the normal feeding process is likely to be very limited. The possibility exists of inducing specific immune protection in the host with parasite molecules other than those presented in normal feeding (Galun, 1975b; Ackerman et al., 1980; Mongi et al., 1986; Willadsen, 1987). The range of potential targets for such artificially primed immunological attack on the parasite by the host is very wide and may be specifically targeted towards disrupting selected tissues or physiological processes. (Essuman et al., 1992).

Immunochemistry And Antibodies As Investigative Tools

Immunohistochemistry techniques are valuable tools employed to detect antigen and antigen sources through the use of specific antibodies that are labelled so that the sites of antibody attachment becomes microscopically visible and still preserve anatomical details. Fluorescent antibody techniques first introduced by Coons et al. (1941) have been used widely on fresh or frozen tissue specimens until recently. These produce labile staining that can be visualized only with an ultraviolet microscope. Enzyme-labelled antibodies and methods applicable to tissues fixed in standard fixtures such as formalin have been more recently being employed in immunohistochemistry. These

techniques create permanent stains that shows the distribution of antigen-antibody complexes under ordinary light microscope (Haines and Chelack, 1991). The underlining principle of these techniques is that labelled antibodies react with antigens without interference with the biological or immunological properties of the proteins. Figure 1.4 shows commonly used immunoenzyme staining methods. Methods in which the primary antibodies specific for the antigen of interest are labelled with an enzyme are termed direct methods (1.4a). In these methods, the antiserum is incubated on the tissue followed by addition of an enzyme substrate that causes the deposition of an insoluble colored reaction product at the sites of antibody binding in the tissues. This reaction product is visible with light microscopy. Direct immunostains are simple and economical to perform; however, they provide little amplification of the visible signal, so they are useful when antigen levels are high. An additional disadvantage is that each primary antiserum must be conjugated to an enzyme.

Indirect immunostain methods utilize an enzyme-conjugated anti-immunoglobulin second antibody to detect binding of the primary antibody to the tissue section (Fig. 1.4b). Although indirect immunostains are somewhat more complex and time consuming to perform, these stains have two advantages over direct methods. Firstly indirect stains enhance the sensitivity of antigen detection because several

secondary antibodies will bind to each primary antibody. This intensifies the visible signal produced by the binding of each primary antibody. Secondly, indirect stains do not require conjugation of each of the primary antisera.

In addition to the indirect methods there are a variety of other immunoenzyme techniques designed for greater amplification of the visible signal produced by the binding of primary antibodies to tissue sections. One of the most versatile of these techniques is the avidin-biotin complex (ABC) method (Fig.1.4c). The ABC immunostain relies upon the high avidity of the B-group vitamin, biotin, for the egg white glycoprotein avidin. Antigens in tissue sections are incubated with an unlabeled primary antibody followed by a second antibody conjugated with biotin. Following exposure to the second antibody, the tissue is subsequently reacted with avidin enzyme substrate complex. Each avidin molecule has binding site for 4 biotin molecules. The avidin-biotin complexes are produced, ensuring free biotin-binding sites on the avidin molecule, which promotes binding of the complexes to tissue-associated biotinylated second antibody. The antibodies are labelled with an enzyme, usually perioxidase. As in direct and indirect immunoenzyme methods, an enzyme substrate is applied to the tissue and a colored reaction product forms on the slide at sites of antibody-enzyme complex binding. The ABC method is technically complex, time consuming and expensive to perform

compared to direct and indirect techniques. However the amplification afforded by this procedure is often necessary to detect antigens in low concentrations or antigens immunogenically altered by formalin fixation.

Analytic immunologic procedures have become important parts of the arsenal of techniques for describing and elucidating physiologic and developmental changes in natural occurring antigens from insects. Antisera, containing specific antibodies have been used as analytic reagents to make qualitative as well as quantitative measurements of these insect antigens. The presence of common or cross-reactive antigens can make specific serological detection or diagnosis of animal exposure to a given agent a difficult task. The development of several immunological and serological techniques have been of great help in the identification and characterization of specific antigens from complex mixtures.

The source of antigen can be located or localized by immunological probing with antibodies. Both monoclonal and polyclonal antibodies can be used for probing. Various immunological techniques have been developed for probing (Kobayashi et al., 1988). The advantage of immunologic detection of antigen is that it does not necessarily require isolation and culture of specific antigens or tissues producing them.

Monoclonal Antibodies

Monoclonal antibodies are homogeneous antibodies derived from a single clone of hybrid cells. They recognize only one epitopes of an antigen (Kennett, 1979; Goding, 1980; Edwards, 1981). Kohler and Milstein (1975) first developed antibody producing hybridoma cells. Antibodies produced in mice, and lymphocytes from the spleen, source of the antibodies, are fused with mouse myeloma cells in culture. The fusion allows the hybrid cells to continue to grow and divide in culture and also to produce antibodies. One hybridoma cell produces one specific antibody. These specific antibody producing cells are selected and cloned.

ELISA (Enzyme-Linked Immunosorbent Assay)

The principle behind the ELISA test is the detection of an antigen with enzyme labelled antibodies (Ma et al., 1988). The enzyme moiety keys a colormetric reaction that is proportional to the amount of antigen molecules bound to the enzyme-antibody conjugate.

There are three ELISA protocols that are frequently employed for the analytic measurement of a chemical in biological samples. They are (1) competitive ELISA, (2) direct double-antibody sandwich ELISA, and (3) indirect antibody sandwich ELISA (Voller et al., 1979).

In the competitive ELISA procedure, enzyme-labelled antigen is used to compete with the nonlabelled antigen

present in a sample preparation for available antibody sites. This protocol is effective if the antigen is stable and can be easily purified in milligram quantities. The amount of bound enzyme-labelled antigen can then be estimated after the addition of enzyme substrate and subsequent colorimetric reading.

The double antibody sandwich ELISA uses an enzyme-labelled antibody that is also specific to the antigen. An antigen molecule is "sandwiched" between the coating or primary antibody and enzyme-labelled secondary antibody.

The indirect double-antibody sandwich ELISA differs from the direct method in that the antigen is sandwiched between specific antibodies developed in two different animal species. If the coating antibody is developed from guinea pig, then the secondary antibody must be from another species such as rabbit. The amount of secondary antibody bound to the antigen is, in turn, detected by a commercially available enzyme-AB conjugate specific for the immunoglobulin of the secondary antibody; in this example amount of bound enzyme-labelled antigen can then be enzyme labelled goat anti-rabbit will act as the tertiary antibody.

Research Objectives:

1. To investigate the attractancy or repellency of hexane washes of cows deemed refractory and susceptible to horn

flies infestation using a ten-port olfactometer.

2. To study the host antibody response to horn fly salivary proteins in naturally exposed mice and immunized rabbits.

3. To identify and characterize immunogenic secretory proteins from horn fly salivary glands.

4. To utilize monospecific polyclonal antibodies produced in rabbits and mice to identify and localilize sites of secretory antigen production *in situ* utilizing immunohistochemical techniques.

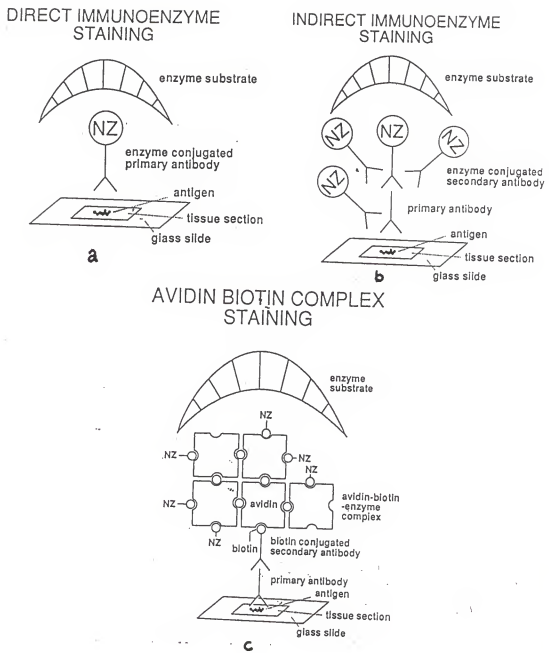

Figure 1.4. Diagrammatic representation of three enzyme immunohistochemical staining methods. a. Direct immunostain. b. Indirect immunoenzyme stain. c. Avidin-Biotin complex (ABC) stain (Haines and Chelack, 1991).

CHAPTER 2
ATTRACTION AND REPELLENT ASSAY FOR TESTING EFFICACY OF HEXANE WASHES OF COWS TO LABORATORY-REARED HORN FLIES USING A TEN-PORT OLFACTOMETER WITH ELECTRONIC MONITORING

Introduction

Blood-feeding insects locate and choose their hosts by responding to a variety of olfactory, visual and thermal stimuli (Vale, 1977; Dalton et al., 1978; Kinzer et al., 1978), but there is evidence that vision is most important (Hargett, 1962; Harget and Goulding, 1962; Milstrey, 1983). About an hour after eclosion, adult horn flies begin to search for a host. Heavy infestations have been observed on dark or black cattle compared to lighter colored cattle (Bruce, 1964). Some animals even of the same hair color, attract more horn flies than other animals (Schreiber and Campbell, 1986; Steelman et al., 1993). Bulls attract more flies than steers or cows (Dobson et al., 1970).

Understanding the factors that influence differential infestation of cows could lead to the development of more effective control measures. Designing laboratory experiments to demonstrate attractiveness or repellency to different odors is difficult, primarily because horn flies exhibit strong phototaxis (Morgan, 1966; Kinzer et al.,

1970), making it difficult to balance light variables in a conventional "Y" tube olfactometer. A "Y" tube olfactometer was successfully used for horn flies by Bolton (1980).

One of the problems encountered in arthropod olfaction studies is the difficulty in measuring and quantifying arthropod behavior to different odors. The "Y" tube olfactometer, which has been used for most olfaction studies, offers flies only two choices (Bolton, 1980). This limits its use in evaluating the response of flies to multiple odors at the same time. Refining or replacing the present methods of evaluating chemicals would be difficult and expensive. Recent technological advancement in electronics and computers, especially in large-scale integration, has resulted in powerful microcomputers at affordable prices that are comparable with, or less than, conventional activity recording equipment (Symonds and Unwin, 1982). Advantages of these systems have helped in the design of an olfactometer capable of testing arthropod response to multiple chemical stimuli without human interference by Dr. J. F. Butler of the Department of Entomology, at the University of Florida (Fig. 2.1) with support from International Flavors and Fragrances, Inc. (IFF, NJ). The patented olfactometer (US Patent: 4,759,228; 5,134,892; 5,118,711; 5,165,026; 5,175,175) is electronically monitored and functions as an activity, feeding, and touch detector by offering individual horn

flies the choice among 10 different odors presented simultaneously on ten artificial hosts. The odors are carried in an airstream moving at 375-500 cm/min. The olfactometer creates distinct and contagious odor fields that can be easily entered, left and reentered by the horn flies seeking a food source. The indices of horn fly response to the odors are recorded on a computer as logged touch and bite contact seconds in a time series of up to 4 hours.

The purpose of this study was to evaluate the response of horn flies to hexane washes of repellent and attractive cows from Arkansas (supplied by Dr. C. D. Steelman through S242 program) and to evaluate the effectiveness of the olfactometer to screen natural chemicals from IFF for future field assays.

Materials And Methods

Individual cows from Arkansas were classified as either resistant (refractory) or susceptible to horn flies by Dr. Steelman, University of Arkansas, based on fly count records. Cows were classified by recording fly populations on individual cows during the summer season for multiple seasons. Hexane extracts of cows were collected during the winter and spring seasons when summer fly pheromone residues would have lost their lasting effect. This period avoids the influence of fly factors which occurs when flies

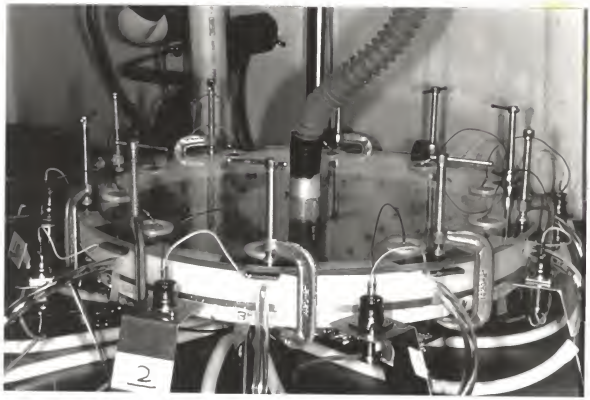

Fig. 2.1. Olfactometer for laboratory assay of hexane washes of attractant and repellent cows against horn flies (UF/IFF Patent No 4,759,228)

are in large populations. A wash of each animal was made by pouring 500 ml of spectograde hexane on the shoulder and side of animals. The rinse was collected in a metal paint roller pan and concentrated at room temperature under NO_2 until the volume was 2-3 ml. The concentrates were put in glass screw cup vials (1.5 cc diameter x 6.5 cm high), covered with aluminum foil, labelled and shipped overnight to the University of Florida where they were stored in an explosion proof refrigerator at 4°C.

Pretests were conducted to identify those animal extracts exhibiting extreme refractoriness or attractiveness to laboratory-reared horn flies. Eight hexane washes were randomly selected from cows classified as refractory or resistant. Another eight were selected from those classified as susceptible or attractive. A comparison was then made between 10 samples made up of 8 cow washes and 2 standards. An attractant standard (IFF.3145) and repellent standard (IFF.3380) supplied by International Flavors and Fragrances (521 West 57 Street, New York, N.Y. 10019).

Attractant and refractory cow washes were compared utilizing the UF/IFF patented pie type olfactometer. Two sets of trials: (1) for the refractory cows and (2) for the susceptible cows were run to select the extreme attractant and extreme refractory cow washes. The eight selected washes from each group along with IFF.3145 and IFF.3380 were randomized within each replication and assigned to the 10

ports of the olfactometer. Each trial was replicated three times. Analysis of variance (ANOVA) statistical analysis (Excel package) was used to select the most active of the susceptible washes and the least attractive in the refractory washes. Four of the most attractive washes and 4 of the least active washes were randomized with the standard attractant (IFF.3145) and standard repellent (IFF.3380) and replicated four times in the olfactometer. After running the washes eight, IFF chemicals identified as repellents were run with the standard attractant (IFF.3145) and untreated air (1000) against horn flies to test the efficiency of the olfactometer in assaying chemicals. Five replicates were run and ANOVA was run on data. Statistics comparison were utilized to determine differences between the standards and the animal extracts at the 4 hour trial period. All trials were run for 4 hours.

The olfactometer chamber acts as a slight attractant for insect movement to the perimeter of the choice chamber. Stimuli other than olfaction, such as light, air flow, temperature of the air and surface, humidity, and detector position, were standardized to aid insect movement to treatment choice sites (UF/IFF pat. No 4,459,228). Because of the ultrasensitivity of horn flies to light differentials, the tests were conducted in total darkness. The olfactometer uses a center point for air exhaust and side ports for presentation of treated air. The odor

sources were presented separately through tygon^{R} (6 mm inside diameter X 17 cm long) tubing attached to the olfactometer and agar block (Fig. 2.3). Agar blocks, sensors, tygon and plate surfaces were replaced after each trial to avoid contamination. Surfaces exposed but not replaced were washed with hot water and sparkleen biodegradable detergent.

Processed air and treatments were introduced through the perimeter ring at 10 ports. Five (5) ul of the hexane washes were pipetted onto a polystyrene disc containing agar covered with a silicone membrane (Butler et al., 1984) and onto polyethylene interflow pellets (Cromex Corp. NY) which were placed within a tygon tube. About 60 to 100 teneral (24-48 hour old) flies of mixed sexes were introduced into the olfactometer after having been immobilized by cooling at 1°C for about 5 minutes. Each trial was run for 4 hours. Fly preference and contact activity were monitored by datalogging the time in bite/contact seconds the flies made contact with sensors (Fig. 2.2), which was then recorded in bits per minute by a computer. Arthropod contact in the olfactometer was monitored and detected by a 10 channel system through Stawberry tree^{R} programming using workbench 3.1 Mac.II (This was specifically adapted to the olfactometer by S.A. Butler, Gainesville, Florida). Strawberry tree program was set up to sense and record feeding through a custom made amplifier (J. Greenberg,

Gainesville, Florida) operating at 100X which detected differential signals produced by the insect feeding through the artificial host (agar block) membrane system (Fig. 2.3). Data were analyzed by logging data into an MS excel spreadsheet utilizing a macro which calculated the total number of seconds flies made biting contact during a given time period (bites second) at 10 or 60 minutes intervals on each channel. The experiments were conducted in a roomsize Faraday cage (Lindgren enclosures, Model No 18-3\5-1) in the dark (Fig. 2.4). Cow washes and chemicals are listed below.

Refractory or resistant cows:

17	271
2876	104
48	1711
3607	33

Susceptible Cows:

H15	36
34	3020
2876a	28
138	17a

Chemicals from IFF

IFF.3620	IFF.3085
IFF.3381	IFF.3022
IFF.3219	IFF.2621
IFF.3218	IFF.2251

Figure 2.2. Horn flies attracted to odor or chemical at a port sitting on sensor.

Figure 2.3. Hook-up of treated artifial host (Agar packet).

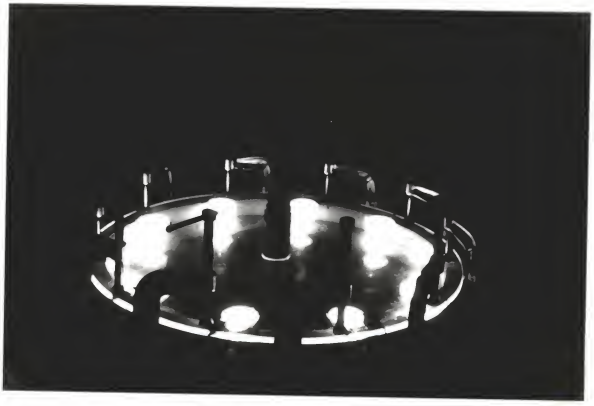

Figure 2.4. Patented olfactometer in operation in the Faraday cage under fiberoptic light conditions.

Results

Figure 2.5 shows the comparison of the IFF standard attractant (3145) and standard repellent (3380) and the gradation of fly activities during the 4 hour runs for the different refractory cow extracts. There was a significant difference in treatment and interaction. Figure 2.6 shows contact second rates for the attractive animal extracts. There was significant differences between replication, interaction and treatment at the P▸0.05. Refractory cow washes tested, with activity close to that of the IFF standard repellent (IFF.3380) (Patent applied for) were considered as strong repellents and selected for further assay. Hexane washes from the susceptible cows tested, with activity similar to that of the known standard attractant (IFF.3145) were considered to be strong attractants and selected for further assay. By ranking, 2876, 33, 1711 and 48 were considered strong repellents and 17a, 28, H15 and 3020 were considered strong attractants.

Results of the trial runs of the combination of the 4 selected strong susceptible washes and the 4 selected strong refractory washes with IFF.3380 and IFF.3145 are shown in Fig 2.7. Hexane wash 2876 maintained its refractory nature. Hexane wash H15 maintained its susceptible or attractant nature. Hexane washes 33 and 1711 reversed their earlier refractory nature and became attractants. Hexane washes 28, 3020 also reversed susceptible nature and became strong

repellents. Washes 48 and 17a which were refractory and attractants respectively, exhibited, near neutral behavior. Figure 2.8 shows the results from the trial of repellent chemicals supplied by IFF. The IFF.3145 was significantly different from all the materials tested. But there was no significant difference between the chemicals tested. The interaction, replication and treatments were also significant. The chemicals and the air (1000) acted as repellents as described by the suppliers IFF.

Discussion

The results of the tests confirms the effectiveness of the multi-port olfactometer as an assaying tool. The results also show that there is a basis for labelling individual cows as susceptible (H15) or refractory (2876) to horn flies. From the tests conducted it is likely that the responsible factors are located in the skin of the cows, but additional tests are required to determine the specific factors involved. Discrepancies occurred with some chemicals reversing roles, especially with the trial combining both attractants and repellents. This may be attributed to the "nearest neighbor effect" and interaction. This means that a stronger attractant may be able to pull flies away from relatively weaker attractants resulting in the relatively weaker attractants acting more neutral or refractory. It may also be attributed to the active

Source of Variation	SS	df	MS	F	P-value	F crit. 0.05
Replication	1498903.01	2	749451.5051	1.544112527	0.21910344	3.097696322
Treatment	31404487.74	9	3489387.527	7.189267026	8.50641E-08	1.985593912
Interaction	44887506.73	18	2493750.374	5.13793243	7.08523E-08	1.71959158
Error	43682461.13	90	485360.6792			
Total	121473358.6	119				

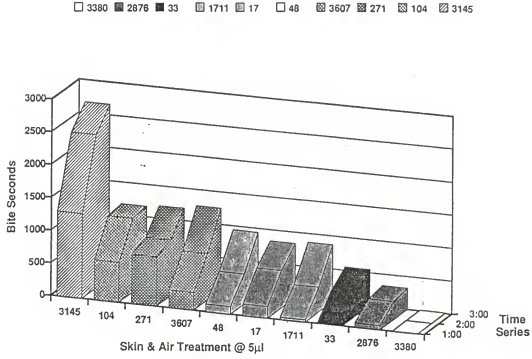

Figure 2.5. Activity of horn flies exposed to hexane washes of resistance or refractory cows. Numbers refer to hexane washes of cows.

Source of Variation	SS	df	MS	F	P-value	F crit. 0.05
Replication	10194833.81	2	5097416.904	22.43507156	5.30862E-08	3.150411487
Treatment	27903921.42	9	3100435.713	13.64583247	1.31044E-11	2.040096092
Interaction	18059874.12	18	1003326.34	4.41590293	6.83283E-06	1.778445835
Error	13632451.02	60	227207.517			
Total	69791080.37	89				

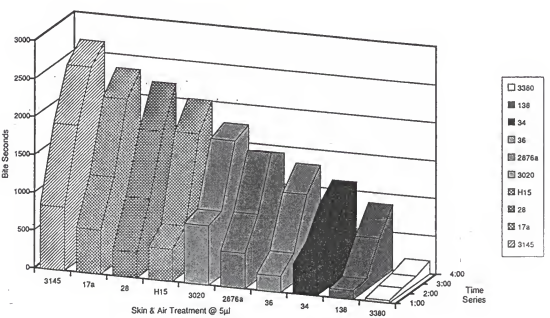

Figure 2.6. Activity of horn flies exposed to hexane washes of cows identified as susceptible or attractive to horn flies. Numbers refer to hexane washes of cows.

Source of Variation	SS	df	MS	F	P-value	F crit. 0.05
Replication	595348482.2	3	198449494.1	32.42537866	2.01469E-15	2.680167199
Treatment	1071100063	9	119011118.1	19.44565588	9.94867E-20	1.958763818
Interaction	2251287057	27	83381002.11	13.6239227	3.27831E-25	1.578923658
Error	734422858.5	120	6120190.488			
Total	4652158461	159				

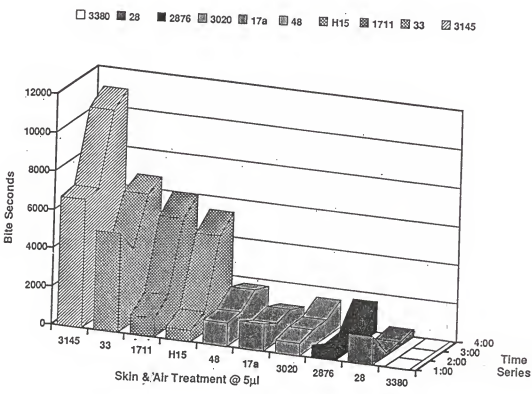

Figure 2.7. Activity of horn flies in the presence of selected refractory cow and susceptible cow washes. Numbers refer to hexane washes of cows.

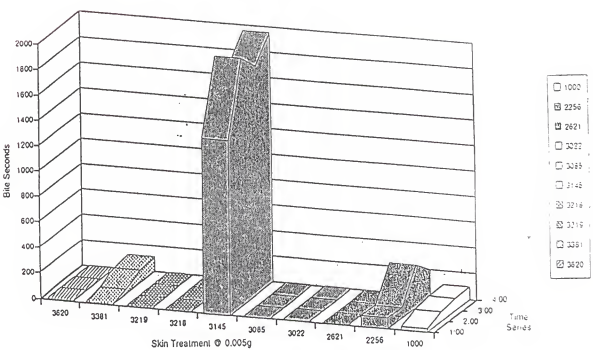

Figure 2.8. Results of testing repellents. Numbers refer to chemicals supplied by IFF.

ingredients in some of the washes breaking down and changing the characteristic of the compound.

In the olfactometer runs, it is possible for different plumes from chemicals or odors offered to the horn flies at different ports to overlap and interact, resulting in the flies making ill-defined chemotactic responses. To avoid this or minimize its effect untreated air was piped between each treatment to keep plumes separate. The untreated air stream also acted as a valve for insects exposed to higher than acceptable odors or chemicals thus acting to reduce attenuation of sensors. This might not occur in a field experiments since the insects will have the option of moving away from odors that are objectionable. To analyze the data from the runs, the total of attractiveness or repellency was logged as a mean over a 4 hour time period. Time of non-touching or non-feeding gaps in the feeding pattern were averaged out with the periods of feeding on bite second intervals at 10 minute or 1 hour blocks.

Testing of the 8 semiochemicals samples from IFF show that they were all highly significant repellents as compared to the attractants. This confirmed the reliability of the olfactometer to screen multiple chemicals for further tests, and helps in avoiding costly and morally challenging use of animal or human subjects. Air (1000) served as a neutral or non-reacting treatment and offered an escape for the flies when overwhelmed or attenuated with repellent chemicals.

Some chemicals are known to maintain a designated fly response description during the initial stages of a trial, then change to an opposite designation with time, this may be ascribed to dosage shifts as materials change or are eliminated or behavioral shifts in the flies. With this observation it will be wise to thoroughly evaluate an attractant or repellent under actual field use before recommending widespread use.

A wide variety of host signals are involved in host finding (Lehane, 1991). The natural refractiveness or attractancy of individual animals to pest infestation has been recognized and has been used to develop livestock lines or breeds with enhanced, genetically based tick and lice resistance (Reik, 1962; Clifford et al., 1967; Wharton, 1974; Sutherst et al., 1979). Horn fly differential infestation of cows, as observed by Steelman et al. (1993) and confirmed by the olfactometer represents an example of natural host resistance to ectoparasitic infestation which need further investigation to determine the factors involved.

Control of an arthropod can be effective only by knowledge of the biology of the target species, in this case the factors used by the horn flies to locate and parasitize their host. Visual and olfactory stimuli, aided by anemotactic and optomotor responses, are considered the most important signals when the insect is still at some distance

from the host. Nearer to the host, different stimuli become important, particularly heat (Lehane, 1991). Despite the importance of individual stimuli, multiple stimuli are likely to be a better guide to the presence of host than one stimuli received alone. It is also possible that horn fly pheromones may influence attack of host (Milstrey, 1983). Horn flies are herd or individual animal parasites, therefore any effect which keeps them on the same host regulates the host parasite interaction.

Understanding all of these factors influencing insect infestation will require the development of new research tools. Successful identification of the factors conferring refractiveness by chemical augmentation, host selection or semiochemical activation could help in the breeding of more resistant animals as part of an integrated control regime. This could reduce cost by reducing the use of chemicals. To determine specifically how the attractancy or repellency of the washes affects the behavior of the flies, further bioassays are needed because experimental design used in bioassay may restrict the nature of stimulus change and fly response. Follow-up field studies are important in confirming laboratory observations. Field and laboratory techniques sometimes give different indications of activity which can be misleading as, for example the range of distance at which flies can detect the washes.

Insecticide impregnated baits made of blends of acetone, 1-octen-3-ol, 4-methylphenol and 3-n-propylphenol are used in Tsetse flies (Glossina spp) control. This control strategy was based on an understanding of the responses of tsetse flies to their host, using research tools that quantify single specific responses (Torr, 1990). Humphreys and Turner (1973) reported that host size is a factor in attracting biting midges Culicoides, but that host color not significant in attracting two species of this genus.

Knowledge of factors conferring repellency to hosts could help to protect man and animals against vector-borne diseases. Animals exhibit strategies to avoid, control or eliminate parasites. These strategies are broadly divided into 5 groups (Hart, 1990). One describes behaviors allowing animals to avoid or minimize exposure to arthropods. The second group control exposure time during which they and their offspring are exposed to parasites. They are thus exposed to small doses of parasites and this helps in the development of immunity. The third group depends on behavioral patterns of anorexia and depression. These are observed when animals get sick with febrile diseases. This potentiates the fever responses enabling the animal to overcome infection. The fourth strategy relates to the tendency of some animals to help group mates or kin that are sick or injured. The fifth strategy is that in

which animals select mates on the basis of evidence of resistance to parasite infection thus producing offsprings with the genetic basis for resisting parasites. Thus the factors conferring refractiveness or resistance of animals are varied, and meticulous studies are needed to identify the most important and influential factors. Currently the innate resistance of cattle to biting arthropods has been masked for several decades by the use of insecticides which have allowed genetically susceptible cows to compete favorably within the herd (Steelman et al., 1993), making it difficult for research into the most important resistance factors. Knowledge has been gained of the behavioral responses of the horn fly to different host odors with this study. The results obtained provides a good starting point for measuring host resistance and for testing chemicals and odors.

CHAPTER 3
CHARACTERIZATION OF IMMUNOGENIC PROTEINS FROM THE SALIVARY GLANDS OF THE HORN FLY, HAEMATOBIA IRRITANS

Introduction

The horn fly is one of the most important pest of cattle in the United States (Marlatt, 1910; Dosey et al., 1962; Bruce, 1964). Losses caused by the fly, the increasing resistance of the fly to the action of insecticides and the high cost of chemicals have stimulated research into alternative control measures of which the possible use of anti-arthropod vaccine is part. Vaccine development will require accurate knowledge of naturally and artificially induced immunity. It is envisaged that antibodies produced in a host by natural or artificial immunization could attach to a wide range of potential targets within an insect, disrupting selected tissues and physiological processes. Wikel (1983) and Willadsen (1980) listed premature detachment, reduced engorgement size, increased mortality, decreased fecundity and diminished hatching in ticks as some of the consequences of host immunity.

Blood feeding ectoparasites present a limited range of antigens to the host with introduction of their saliva (Nelson et al., 1977). The host's immune system, recognizing these antigens in the saliva, produces antibodies to counteract them. When the antibodies are taken in the blood meal by hematophagous insects, they bind to their antigenic counterparts within the insect, and may disrupt cellular function and increase mortality. Host antibodies interfere with normal tick feeding by inhibiting the activities of secreted salivary enzymes (Reich and Zorzopulus, 1980). Anti-tick vaccine has been produced and successfully used to protect cows. A variety of extracts from tissues which normally have no contact with the host induce immune protection to tick infestation (Galun, 1975b; Ackerman et al., 1980; Mongi et al., 1986; Willadsen, 1987).

Identification and characterization of appropriate antigen(s) and the mechanism of presenting these antigens to the host in a manner that stimulates the proper long lasting immune response is important in the development of a vaccine. Identification of antigens in blood-feeding insects requires knowledge of the functions of saliva, since it is often introduced into host. There is little knowledge of the function of saliva in horn flies and many other important ectoparasites.

Various roles have been ascribed to the saliva of hematophagous arthropods, though until recently no general

role for saliva in hematophagy was apparent (Ribeiro, 1987). Some blood-feeding insects feed in the absence of saliva (Lester and Lloyd, 1928; Hudson et al., 1960; Hudson, 1964; Rossignol and Spielman, 1982). Saliva also functions in ways unrelated to blood-feeding (Ribeiro, 1987). It is involved in dissolution of solid sugar (Eliason, 1963) and in lubrication of the feeding stylets in mosquitoes (Orr et al., 1961). Gooding (1972) found anticoagulants and hemagglutinins in the saliva or salivary gland homogenates of some blood-feeding arthropods but could not ascribe a role to them. Kerlin and Hughes (1992) reported that salivary secretions from parasites probably were produced to promote parasite feeding and survival on the host. Saliva from Ixodes damini contain anti-haemostatic, immunosuppressive, anti-inflammatory, and neutrophil-inhibiting components (Ribeiro et al., 1985). Saliva of some hard ticks contain prostaglandins that may help feeding by increasing host skin circulation at the site of the bite (Binnington and Kemp, 1980; Kemp et al., 1982). Their salivary apparatus also contributes to ion and water metabolism by excreting excess water acquired from the blood meal (Binnington et al., 1980; Kemp et al., 1982).

Saliva from blood-sucking insects contains toxins or allergens (Gothe et al., 1979; Wikel, 1982; Wikel and Allen, 1982). Host allergic or hypersensitive response to saliva introduction functions to diminish arthropod feeding success

(Wikel and Allen, 1982). Repeated exposure to salivary antigens may cause a host to produce antibodies that may alter the feeding site of the arthropod (Wikel, 1982; Wikel and Allen, 1982).

Any vaccine development must consider several questions. These include the type of effector cell involved in resistance, the protective antigens that are recognized, and the form in which these exogenous proteins are presented.

The purpose of this work is to identify and characterize antigens of potential value. This study attempts to identify antigens from the salivary gland and salivary secretion of the horn fly using Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Western blots of SGE were reacted with rabbit anti-salivary gland extract and mouse anti-salivary secretion serum to identify proteins bands of immunological importance.

Materials and Methods

Horn Fly Rearing And Culture Procedures

Horn fly (Haematobia irritans (L.)) adults were obtained from the laboratory colony at the University of Florida (Greer, 1975; Bolton, 1980). Standard colony rearing methods were modified from those of Greer (1975) as follows: A larval medium was made from a mixture of 1370 g of cattle manure (frozen at -20°C for at least 48 hours to kill all naturally occurring dung parasites), 546 g

pelletized peanut hulls (High Springs Milling Co Ltd., High Springs, FL.) and 258 g dry mix (44% all-purpose flour, 33% fish meal, 18% alfalfa meal, and 5% baking soda). Peanut hulls were presoaked in 700 ml of water for 30 minutes, and then crushed by hand. The dry mix was added to the peanut hulls and mixed thoroughly before the manure was mixed in. Horn fly eggs (0.5 ml) were added to about 1400 g of medium, which was maintained for 7 days in an environmental chamber (Percival^{R} Boone, Iowa) at 27 ± 3°C and 75 ± 5% R.H., with continuous light supplied by two fluorescent tubes. Pupae were separated from the medium by water floatation and dried on paper toweling. Dry pupae were held in a screen-covered aluminum cage (51 X 26 X 27 cm) in an environmental chamber until adults eclosed.

Adults were fed whole bovine blood obtained from freshly slaughtered cows. One liter of blood was collected in a 2.5 liter vessel containing 3.96 g of sodium citrate in 13.2 ml of water. Kantrex^{R} powder (Apothecon, Princeton, NJ) (0.24 g) and 0.022 g of Mycostatin R (E. R. Squibb & Sons, Inc. Princeton, NJ) powder were added to 1 liter of citrated bovine blood to control microorganisms and prevent spoilage. The blood was stored at about 4°C until needed. Beef juice, the fluid drawn off from stored cut or ground meat was obtained locally and was added to the citrated bovine blood at a ratio of 75 parts blood:1 part beef juice.

Dissection Of Horn Fly Salivary Glands For Antigen Production

Salivary glands were surgically removed by the following protocol. Groups of teneral adult flies (24-48 hours old) were immobilized by cooling for about 20 minutes at 1°C. The immobilized flies were glued dorsally with Super Glue® onto wax strips demarcated 3 X 1 inch glass slides. The flies were flooded with insect ringers solution (Smith and Butler, 1991). Legs were first removed with a fine tipped forceps, the ventral integument was then removed anterior-posteriorly from the thorax to the abdomen. The exposed salivary glands were teased out and removed with the forceps (Fig. 3.1). The glands were washed and stored in insect Ringers solution containing 10 mM of Phosphonylmethyl-sulfonyl fluoridate (PMSF) kept on ice. PMSF phosphorylates the active site of serine proteases and inhibits their activity. The salivary glands were homogenized in a Tenbroek tissue homogenizer, sonicated (55,000 cycles/second) in 30 second bursts on ice and centrifuged at 13,000 g for 20 minutes to remove insoluble material. The supernatant was decanted and was referred to as salivary gland extract (SGE). The SGE was divided and stored at -20°C until used. The concentration of protein in the SGE sample was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as standard.

Polyclonal Antibody Production.

Two New Zealand female white rabbits (3-4 months old) and two female ICR mice were used as models to generate antibodies against horn fly salivary components. The mice and rabbits were kept in individual cages fed a pelleted diet and water ad libitum, and kept on a 12:12 light-dark cycle. Care was taken to prevent ectoparasitic infection of the animals.

Two different methods utilizing two antigen sources and two different host models were used for antibody production. These were: (1) Immunization of rabbits with SGE with Freund's adjuvant and (2) Naturally infesting caged restrained mice (Fig. 3.2) by exposing these mice to horn fly bites. By this method salivary secretions associated with horn fly blood feeding served as the immunogen.

(1) The immunization with the crude SGE was contracted to Kel, Inc. Gainesville, Florida, who performed the following immunization and bleeding protocols. Preimmune blood samples from the unimmunized animals were taken prior to immunizations. A volume (1ml) of SGE antigen (2 mg/ml), was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneosly into each of the rabbits. This was followed by bi-weekly immunizations of antigens in Freund's incomplete adjuvant. On day thirty, sixty and ninety post immunization (PI) blood samples were collected by cardiac puncture from the rabbits. The

collected blood was clotted overnight. Serum was separated by centrifugation, decanted, pooled and stored in small aliquots at -20°C until needed.

(2) Preimmune serum was obtained from the mice before infestation. Two mice were cage restrained (Fig. 3.2) and sequentially exposed twice weekly to (24-48 hr old) teneral horn flies. The immobilized mice were placed in a cage containing about 100 horn flies of both sexes. On days 30, 60 and 90 antisera were obtained from the mice by cardiac puncture. The blood was allowed to clot. The clotted blood was then centrifuged at 20,000 g for 15 minutes and serum obtained. The serum from each experimental group was pooled. The pooled antisera was stored in aliquots at -20°C until used. The sera from the two model animals were tested for antibody production with ELISA. When a high antibody titer had been obtained animals were sacrificed and antisera was obtained for tests.

DOT-Enzyme-Linked Immunosorbent Assay (DOT-ELISA) For Determining Optimum Antigen Titer.

Assay was performed using serial dilutions of the SGE stock solution in PBS containing from 1 ug/ml to 20 ug/ml. The serially diluted (2 ul) samples were pipetted individually onto spots on two (20 X 10 cm) PVDF (Polyvinylidene difluoride) protein sequencing membranes. The PVDF membrane had previously been presoaked with absolute methanol. Two dots of PBS/T were used as a

negative control and another two dots which had antigen but treated with normal mouse serum (NMS) and normal rabbit serum (NRS) were used as positive control. The PVDF membranes were handled with gloved hands and forceps to prevent contamination. The dotted PVDF membranes were immersed in 3% BSA-PBS-T (3% bovine serum albumin-0.01 M phosphate buffered saline containing Tween 20), washed in PBS-T (phosphate buffered saline containing Tween 20) drying agents and air dried. Two microliters of serially diluted rabbit anti-SGE serum and mouse anti-salivary secretion serum (1:50 to 1:3200) were applied on top of the dots on the two membranes, respectively. After 30 min incubation, the PVDF membrane were washed 3-5 times in PBS-T, soaked in alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG at 1:1000 dilution, respectively, and left to react at room temperature for 30 minutes. The PVDF membranes were washed three times in PBS-T, once in PBS, and immersed in substrate solution, freshly prepared by mixing equal volumes of BCIP and NBT. After color development the PVDF membrane was then washed with water and air dried. The intensity of the yellow color was visually compared in reference to the negative and positive controls.

Antibody Titer Determination Using Enzyme-Linked Immunosorbent Assay (ELISA)

An ELISA was used to determine the titer of model animals antibody response to immunization with SGE and

natural horn fly infestation. To determine optimum antibody titers, pooled serum from the rabbits immunized with SGE and naturally exposed mice were assayed on days 30, 60 and 90.

One hundred uls of a working dilution of 2 ug/ml per well (determined as optimum by DOT blot ELISA assay) of SGE diluted in PBS (pH 9.5) was placed in a 96 well flat-bottomed Nunc Maxi-sorp, immunoassay plates (Dynatech, Chantilly, VA) to coat the plates. Positive controls containing antigen but reacted with NMS and NRS and negative controls containing (PBS) were run simultaneously. The negative control was used to give background optical density. The plate was incubated overnight at 4°C. Following binding of the antigen to the plates, the plates were washed 4X with wash buffer (PBS with 0.02% sodium azide, 0.05% Tween 20). The non-specific binding sites were blocked by adding 300 ul/well of blocking buffer (1% BSA in PBS with 0.02% Sodium azide) to the microtiter plate and incubating the plate for 60 minutes at room temperature. These were washed 4X with wash buffer as described above.

Wells were then incubated with 100 ul/well of serially diluted (1:50 to 1:3200) rabbit anti-SGE, and mouse anti-salivary secretion serum. The wells were incubated for 60 minutes at room temperature and were then washed with wash buffer as described previously. One hundred ul/well of rabbit anti-mouse IgG whole molecule alkaline phosphatase conjugated (Sigma) diluted (1:1000) in PBS with 0.02% azide,

and goat anti-rabbit IgG whole molecule alkaline phosphatase conjugated (1:1000) were added to the wells containing the respective primary antibodies as secondary antibody and incubated at room temperature for 60 minutes. The wells were then washed as described above. One hundred ul/well of 1 mg/ml freshly made substrate, Para-nitrophenyl phosphate (Sigma) in diethanolamine substrate buffer (Sigma Chemical Co., St. Louis. MO) with 1 mM $MgCl_2$. pH 9.8) was added to the plates and incubated for 60 minutes at room temperature in the dark. Color development was stopped by the addition of 50 ul/well of 3M NaOH. The absorbance was read at 405 nM at 30 and 60 minute time intervals on a Dynatech Micro ELISA reader (Dynatech Laboratories).

Protein Separation With Polyacrylamide Gel Electrophoresis

SGE antigens and known protein standards were electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% separating gel preparation with 4% stacking gels (Bio-Rad Laboratories) (Laemmli, 1970) Protein bands were visualized with coommassie brilliant blue stain. The molecular weight of separated proteins was estimated by comparison with electrophretically separated low molecular weight and high molecular standards (Bio-Rad laboratories, 32nd and Griffin Ave., Richmond, Calif.).

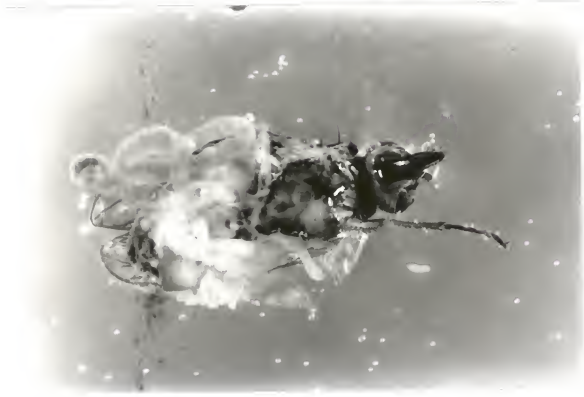

Figure 3.1. Dissection of the salivary glands of the horn fly.

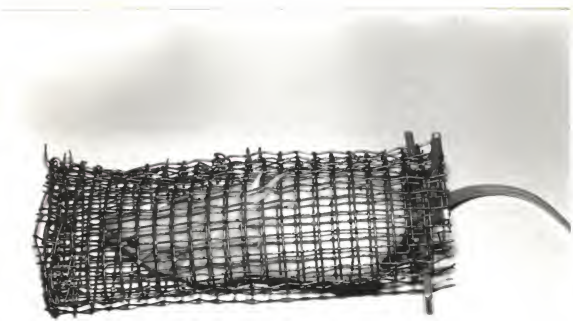

Figure 3.2. Restrained mouse ready to be offered to horn flies for feeding

Twenty (20) ul of SGE sample containing 5-20 ug/ml of soluble protein were diluted in equal volume of 2X sample buffer (0.08 M Tris-HCL, pH 6.8, 0.1 M dithiothritol, 2% SDS, 10% glycerol, 10% 2-mercaptoethanol, 0.2% bromophenol blue) and heated in a water bath at 100°C for 4 min. These prepared samples were loaded into the sample wells (10-20 ul). Molecular weight of electrophoretically separated protein bands were estimated by measuring the migration distance of protein bands against prestained low molecular weight standards (Phosphorylase b, Mr=130KDa; Bovine serum albumin (BSA), Mr=75KDa; Ovalbumin, Mr=50KDa; Carbonic anhydrase, Mr=39KDa; Soyabean trypsin inhibitor, Mr=27KDa and Lysozyme, Mr=17KDa) included with each electrophoresis run. Bromophenol blue incorporated in the buffer allowed monitoring of protein migration during electrophoresis. Two slab gels were run concurrently. For optimum protein separation with 2 gels, voltage was held constant at 200 v until the sample migrated into the separating gel. When the bromophenol blue dye front was about 2 mm from bottom of the gel the voltage was shut off. Gels were fixed and protein bands were visualized by staining with 0.1% coomassie brilliant blue R-250/40% methanol/acetic acid for 2 hours and destained with 40% methanol/10% acetic acid.

Western Blot

Following SDS-PAGE of the SGE, the unfixed resolved proteins were transferred electrophoretically onto PVDF Immobilon transfer membrane (Millipore Corp. Bedford, Mass) employing the method of Towbin et al. (1979). Proteins were transferred from the gel to the PVDF protein sequencing membrane at constant voltage (25 V) overnight at room temperature. The gel and PVDF blot were removed from the cassette once the transfer was complete. The gel was stained with 1% coomassie blue to ascertain if the proteins were adequately transferred. The PVDF protein sequencing membrane were equilibrated in Tris buffered saline (TBS) for 30 minutes on a rocker and, then, blocked in a solution containing bovine serum albumin and fetal calf serum (3% BSA, 5% fetal calf serum, 0.1% Tris buffered saline with Tween 20) for one hour. The PVDF membrane were transferred to TTBS for 2 successive washes (5 mins each) and air dried. It was then put in a zip lock bag and frozen at -20oC until used. The PVDF blot was cut into 1 cm vertical strips corresponding to the individual lanes of migrating protein.

Immunodetection Of Salivary Gland Antigens

The affinity of the pooled antisera from the two model animals for the electrophoretically separated and immobilized SGE antigens was tested. The methodology used was a modification of that described by Towbin et al.

(1979). Two strips of the blotted PVDF membrane were socked in bovine serum albumin and fetal calf serum block for one hour. This was then washed 3 times with TTBS for about 5 minutes each. The two strips were then incubated either with the polyclonal primary antibodies from the rabbit or the mouse for 3 hours at 1:200 dilution in TBS (found to be optimum with ELISA). The strips were then washed 3 times for 5 minutes each in Tris buffered saline and 0.5% tween 20 (TTBS) to remove the unbound antiserum. The attached mouse antibody was labelled by alkaline phospatase attached to goat anti-mouse secondary antibody (1:1000 in block dilution) and the rabbit antibody was labelled by alkaline phosphatase attached to goat anti-rabbit secondary antibody for 1-2 hours. These were used to visualize the SDS-PAGE separated SGE proteins. The blots were removed and washed 3X for 5 minutes each with TTBS. Color development was achieved by placing the blots in 0.1 M Tris-HCl buffer, 10 mM $MgCl_2$ pH 8.8 for 15 minutes. This was decanted and the substrate BCIP/NBT (5-bromo-4-chloro-3-indoxyl phosphate and nitrozolium blue (sigma) (50 ml of 0.1 Tis/MgCl2 solution pH 8.8, 50 ul of BCIP and 100 ul NBT) was added. Color development was stopped by immersion in distilled water for two 10 minutes washes. The strips were dried between filter paper and photographed.

Evaluation Of SGE For Carbohydrate Epitopes

Periodate oxidation reaction was used with ELISA technique to evaluate the reaction of the mouse and rabbit polyclonal antibodies to SGE as to reaction to carbohydrate or non-carbohydrate determinants (Woodward et al., 1985). To identify the type of epitope found in SGE, ELISA titers of periodate treated and non-treated SGE were compared. Two microtiter plates were used, one for the periodate assay and the other used as control. Fifty microliters of a working dilution of 2 ul/ml of SGE diluted in PBS (pH 9.5) was added to the microtiter plates. The plates were incubated overnight at 4°C. The plates were then washed 4X with wash buffer (1X pbs with 0.02% sodium azide, 0.05% Tween 20). Non-specific binding sites were blocked by adding a blocking buffer 1% BSA (Bovine Serum Albumin) in PBS with 0.02% sodium azide and incubating in block for 60 minutes at room temperature. The wells were then washed with the buffer described above. Each microtiter plates was then washed with 50 mM sodium acetate buffer (pH 4.5). One microtiter plate was incubated with 100 ul/well of 10 mM sodium meta-periodate (Sigma Co. St. Louis, Mo) in 50 mM sodium acetate buffer, pH 4.5, for an hour at 25°C in the dark. Following a brief rinse with 50 mM sodium acetate both plates were incubated with 100 ul/well 5 mM sodium borohydride (Fisher Scientific, Fairlawn, NJ) in PBS for 30 minutes at 25°C. The reagents used were freshly prepared just before use.

After 4 washes with wash buffer the ELISA procedures were performed as described previously.

RESULTS

Optimum Antigen And Antibody Titers

An optimum concentration of 2 ug/ml of SGE per dot allowed detection by both polyclonal antibodies at 1:200 serum dilution. This concentration of antigen was therefore used to coat ELISA plates. ELISA reading of antisera from immunized rabbits and ICR mice showed that high antibody production was stimulated about 60 days after immunization. The results of the ELISA experiment with pooled mouse and rabbit antisera at day 60 post immunization and baseline serum are shown in Fig. 3.3. With positive controls (antigen reacted with Normal mouse serum (NMS) and normal rabbit serum (NRS)) as base line, the titer of the anti-SGE response and the mouse anti-salivary secretion response are indicated as the last dilution of sera with an optical density of 0.05 higher than the base line. Mean OD for rabbits ranged from 0.5 to 2.25. That for mice ranged from 0.25 to 0.5. The optical density decreased with increasing dilution of the sera samples. Low absorbance values (less than 0.05) were obtained with the negative controls. The titer of both the mouse and rabbit antisera was determined to be 1/200 dilution. At high dilutions, antibody was still detected by the ELISA procedures (1/6400 by the rabbit

antisera and 1/3200 for mouse antisera). There was a higher antisera titer in the rabbit compared to the mice. From these results it can be seen that all infected animals showed antibody titer significantly ($p=0.05$) above that found in uninfected animals and the antibody generation between the two procedures was also significant.

Polyacrylamide Gel Electrophoresis, Western Blot And Characterization Of Salivary Gland Aantigens

Denaturing, discontinuous SDS-PAGE analysis as described by Laemli (1970) was used to analyze the proteins in the SGE from the horn fly. The SGE separated into about 12 bands (Fig. 3.4). Readily detected proteins range in molecular weight from about 75 KDa to less than 14 KDa. Three prominent or major bands were observed consistently at about 27, 50 and 75 KDa. Western blot analysis of the SGE using the pooled diluted (1/200) sera from the rabbits and the mouse show that the mouse antisera recognized 2 protein bands, one major one and a minor one at about 27 KDa (Fig. 3.5). Seven reactive bands were precipitated by the rabbit anti-SGE. A major band was recognized just below 30 KDa. Two bands were just below 21.5 KDa, three bands between 46 and 69 KDa and a band at about 200 KDa. Normal rabbit and mouse serum had no reactivity to salivary gland antigen (Fig. 3.6 and 3.8 respectively). The proteins were characterized according to molecular weight by comparing their relative mobilities on SDS-PAGE with those of known

molecular weight standards. Both sets of sera consistently reacted with the major protein band at about 27 KDa. This was therefore identified as a secretory protein.

Periodate Oxidation

Antibody binding of the SGE antigen treated with meta-periodate was reduced by sera from both host models. The reduced antigen-antibody reactivity resulted from the oxidation of the carbohydrate epitopes by the sodium meta-periodate. The OD level of the rabbit antibody reaction was reduced by a greater percentage by the periodate reaction compared to the reduction of the mouse antibody reaction (Table 3.1). This results shows that the polyclonal antibodies recognized carbohydrate-conjugated epitopes associated with glycoproteins making up the SGE antigen.

Discussion

The need for adequate quantities of high quality antiserum in immunological investigations cannot be underestimated. The two procedures (immunization and natural infestation) used for the antibody generation in the model animals enabled a response to both the potential and "true" salivary antigens to be compared (Cross et al., 1993). Using the procedures also overcome the difficulty in obtaining pure and adequate horn fly salivary antigen for

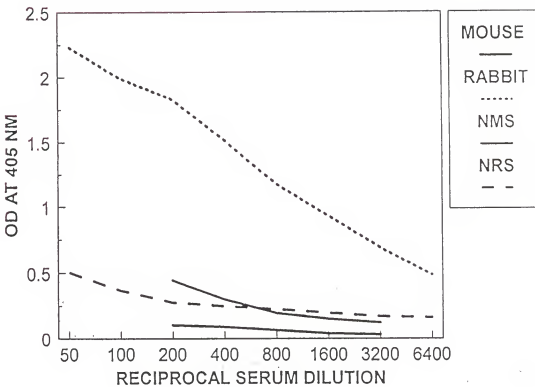

Figure 3.3. Elisa showing binding efficiency of rabbit anti-SGE and mouse anti-salivary secretion sera. Secondary antibodies were coupled to alkaline phospatase and presence of bound antibody was detected colormetrically using p-nitrophenylphosphate as a substrate.
(representative data from 3 replicate experiments are shown).

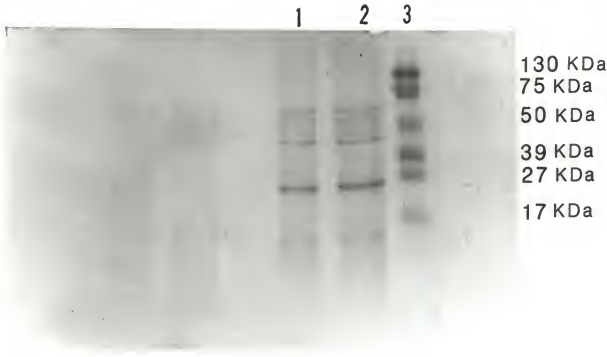

Figure 3.4. Photomicrograph of 12% SDS gel, depicting the protein profiles of salivary gland extract from the horn fly stained with coomassie blue. Lanes 1 and 2 show the proteins in the SGE. Lane 3 shows the molecular weight markers.

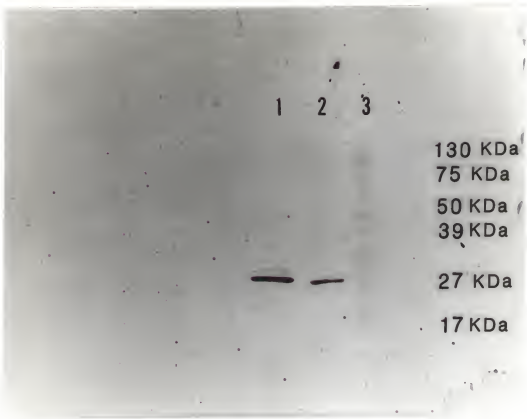

Figure 3.5. Western blot of SGE exposed to mouse anti-salivary secretion antibody and visualized with alkaline phospatase labelled secondary antibody. The mouse serum precipitates a broad band at approximately 27 KDa. Lane 1 and 2 the immunoprecipitated bands and lane 3 shows the molecular weight markers.

Figure 3.6. Immunoblot of SGE exposed to NMS (Normal mouse serum). Note the lack of immunoprecipitation.

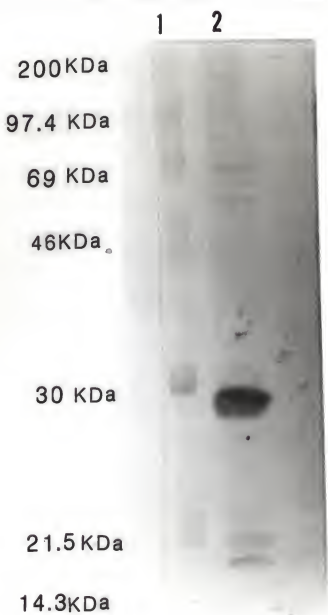

Figure 3.7: Immunoblot of SGE exposed to rabbit anti-SGE serum. Lane 1 shows the molecular weight markers. Lane 2 shows the precipitated bands. Note the broad band just below 30 KDa.

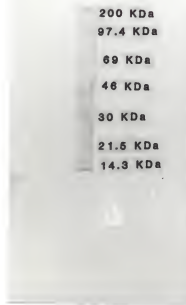

Figure 3.8. Immunoblot of SGE exposed to NRS (Normal rabbit serum). Note the absence of any precipitation.

Table 3.1. Optical Densities Of Periodate Treated SGE And Binding Of Mouse And Rabbit Antibody.

Treatment	Antibody	OD (405)[a] nm
Control[b]	Rabbit anti-SGE	2.06
SGE + periodate	Rabbit anti-SGE	0.13
Control	Mouse anti-SSc	0.61
SGE + periodate	Mouse anti-SS	0.03

[a]Results were averages from 3 readings.
[b]The controls were treated with buffer (acetic acid-sodium acetate at pH 4.50 followed by sodium borohydride.
[c] Antibody from mice against the salivary secretion associated with horn fly feeding on mice.

immunological studies, it also provided a basis for comparing the effect of the two procedures. The results of the ELISA (Fig. 3.3) showed that the model hosts developed an immune response to antigens from the salivary glands of horn flies. This is shown by the development of high antibody levels in the animals. Consistent increases in concentration of immunoglobulin was observed for both model animals but different antibody levels were obtained for both. The high antibody titer observed with the ELISA with the two model animals showed that the two laboratory animals could be used for the study of host immune response to horn fly salivary antigens. The lower antibody levels developed in the mice exposed to horn fly feeding could be attributed to the horn flies not producing enough critical mass antigen to elicit adequate antibodies. The differences in the antibody levels generated in the model hosts may reflect variations in the response of the two different hosts to the different antigens and also the different modes of immunizations employed. Ribeiro (1989) reported differences in relative amounts of anaphylactic response mediators among vertebrate hosts. Despite the low antibody titer of the mouse, it was able to immunoprecipitate protein bands later on. One advantage of a high antibody titer in immunological studies is that it allows for a high antisera dilution which dilutes out population of unwanted or nonspecific antibodies that might interfere in immunological work. This allows for

efficient use of antisera and allow for the fullest use of the available quantity of high antibody, which often is expensive and difficult to produce (Polak and Van Noorden, 1984). One of the most important attributes of good quality antibody is its high affinity for its antigen, its binding sites should fit well with the antigenic sites on its specific antigen and not attach to other antigens (Polak and Van Noorden, 1984). The affinity of the SGE and the antisera from the two model animals seems good but the possibility exists to obtain better affinity with pure horn fly salivary antigen. This cannot be presently compared because of the failure to obtain adequate quantities of horn fly salivary secretion. To enhance antibody production intra splenic immunization or in vitro methods could be used. Different methods have been employed to obtain saliva from arthropods. Oral secretions of fleas has been collected directly into containers (Michaeli et al., 1966), those of mosquitoes into distilled water (Allen and West, 1966), or those of ticks into capillary tubes (Clarke and Hewetson, 1971). The buffalo fly, Haematobia irritans exigua was induced to salivate with 80 mM serotonin (5-hydroxytryptamine) in saline but very minute quantities were obtained. Even with pure antigens, the antiserum produced cannot be guaranteed to be directed specifically and solely to the injected antigen (Polak and Van Noorden, 1984). The antibodies produced could be directed to various parts of

the antigen molecule and to the carrier protein, or part of it. Elisa provides a good screening test for antibodies and can be used to check for cross reactivity or contaminating antibodies (Polak and Van Nooren, 1984).

In the development of an anti-arthropod vaccine, an understanding of the structure and function of the antigen and the mechanism of the host response are essential. The use of vaccines made up of antigens from the saliva and other tissues has been shown to be effective in controlling ticks (Wikel et al., 1992; Agbede and Kemp, 1986). The possibility of exploiting the host immune response to control flies was investigated by Schlein and Lewis (1976). They demonstrated that hematophagous flies exhibit increased mortality and cuticular abnormalities when allowed to feed on rabbits immunized with fly extracts or fly integuments or other tissues. Detailed studies of the horn fly salivary antigen and proteins that can induce immunity in host are essential processes in the development of an anti-arthropod vaccine. The results of the SDS-PAGE demonstrated that SGE is made up of multiple proteins (Fig. 3.4). Probing of the western blot of the SGE with the mouse antisera precipitated two bands (Fig. 3.5), and the rabbit antiserum precipitated 7 bands (Fig. 3.7). The consistent identification of a common band at about 27 KDa by both antisera leads to the conclusion that the protein is secretory and warrants detailed studies for its suitability as a potential antigen

for vaccine production. The immunoprecipitation of fewer protein bands by antisera from the naturally exposed mouse as opposed to the immunized rabbit is not unusual. Similar results were observed in work with tick salivary antigen and black fly salivary antigens (Cross et al., 1993; Nyindo et al., 1989). The results probably reflect variations in the antigen used and the mode of immunization. Variation in the results is important because it may be due to low level of expression of the salivary secretion when introduced by the horn fly as opposed to the high level of expression when introduced through immunization. The identification of one protein band as secretory antigen may also indicate that only few antigens are present in the salivary secretion of the horn fly or that few antigens in the saliva generates an immune response. It has been observed in other studies that antibodies were not detected against molecules corresponding to the molecular weight of substances known to be pharmacologically active in black fly saliva (Jacobs et al., 1990; Wirtz, 1990). This indicates that the host does not mount a response which would potentially interfere with the functions of these components (Cross et al., 1993). The periodate study indicates that the SGE possesses carbohydrate antigenic determinants which may be involved in antibody binding.

Understanding of the immune reaction of immunized hosts and naturally exposed host could help in the identification

of antigens to be used in a vaccine and the form in which it should be presented to host to obtain resistance. The likelihood that the horn fly salivary antigen changes during feeding makes the study of salivary heterogeneity important. Heterogeneity of salivary gland secretion during feeding has been observed in ticks. The size, mass and protein content of the salivary gland increased about 25 fold during feeding (McSwain et al., 1982). Kerlin and Allingham (1992) demonstrated that cows do not exhibit immunity to buffalo fly attack even when high levels of antibodies to buffalo fly salivary antigens have been recorded. They concluded that buffalo fly salivary antigens could not be good candidates for vaccine production. On the contrary it has been demonstrated that tick anti-saliva antigen imparted immunity onto the host (Brown and Askenase, 1986; Shapiro et al., 1989; Jaworski et al., 1990). Nelson et al. (1977) reported that the active fraction in an extract injected may be infinitesimal, or may require a complementary substance to induce sensitization. Thus the host could not possibly be able to detect all the antigens in the saliva, since it is possible that only a small fraction of the salivary components are active. The preparation of the SGE antigen may also affect results obtained. This is because the temperature requirement for active secretion and action of the saliva may be incompatible with processing and preservation procedures used in this study. The insect may

also secrete a substance that cannot be found in the extract. The seven bands identified by the rabbit sera could be potential antigens and further study is required to elucidate this. The presence of the homologous 27 KDa band shows that at least some of the active ingredients in the saliva had been captured by the SGE preparation.

The identification of antigens from the saliva of the horn fly using rabbits and mice as models was based on the concept that immunizing hosts with vaccines composed of those antigens would elicit antibodies, which when ingested by the fly would bind to target tissues, producing deleterious effects. Tick salivary gland immunogens are responsible for stimulating and interacting with the host immune response. The lack of any demonstrated protective function for host immunoglobulin against many hematophagous insects indicates the need for further research to produce anti-arthropod vaccine.

Salivary antigens may be poor antigens. The horn fly also may have the ability to evade or depress the host's immune system to ensure feeding success. Wikel and Whelen (1976) and Barriga et al. (1991) demonstrated that tick infestation depresses the host's immune response. This is done in part by inhibiting the production of effective T cell or specific antibodies. Horn flies produce little quantities of saliva as compared to stable flies or ticks (Butler et al., 1977). They also reported that as a result

regurgitative transmission of disease was less likely in horn flies as compared to stable flies because the horn fly produces little saliva as compared to say the tick. The possibility of horn fly immunosuppression of host immune response makes the study of antigens from other tissues that are not normally in contact with very important since this has been demonstrated in ticks. The mechanism of host resistance to ectoparasites and the effects of the resistance on ectoparasites is not fully understood. Nelson and Kozub (1980) observed that acquired resistance to sheep ked was a local phenomenon that develops in infested areas of skin and is not observed in other areas of skin in the same animal. Allen and Nelson (1982) suggested that chronic inflammatory reactions may be responsible for the localized nature of resistance.

The general approach to vaccine production was summarized by Zanetti et al. (1987) who recommended that the best method to demonstrate antigens of potential use in a vaccine is to identify the molecules that produce immunodominant response in sera from infected individuals.

CHAPTER 4

DETECTION OF ANTIGEN SOURCE USING IMMUNOHISTOCHEMISTRY

Introduction

In the development of an anti-arthropod vaccine one of the most critical steps is the identification of target tissues producing immunogens. This is very important in understanding the relationship between immune response and pathology in the arthropod, because the aim of an anti-arthropod vaccine is to use appropriate arthropod antigens to elicit host antibodies responses. The feeding arthropod ingests the antibodies with its blood meal, which binds target tissues producing deleterious effects. When stable flies, Stomoxys calcitrans (L.) fed on rabbits immunized with selected tissues from the flies they developed ill-effects including increase in mortality. The rabbit antibodies passed through the gut and directly affected fly tissue (Schlein and Lewis, 1976). Different techniques exists for the identification of antigen source. In the present study immunohistochemical immunostaining technique was most appropriate. In immunohistochemical staining antigens are detected in tissue sections through the use of labelled specific antibodies. This makes the sites of antibody attachment visible microscopically (Haines and

(Chelack, 1991). The specific immunostaining technique used in this study was the avidin-biotin complex (ABC) method. It affords amplification that enables detection of minute quantities of antigens or antigens immunogenically altered by formalin fixation. For successful immunostaining, the tissue antigens must be insoluble but still have antigenic sites available for attachement by antibody without great alteration of their tertiary structure. In addition, the anatomical details of tissues must be preserved (fixed) so that the immunoreactive cell or organelle may be identified in context. Secondary antibodies labelled with perioxidase or alkaline phosphatase as in this study have the advantage that an ordinary transmitted-light microscope can be used and the preparations are permanent. The development of the enzyme reaction in this case is progressive and can be monitored. Most immunological studies isolate and characterize arthropod antigens, but in only few cases have studies directly showed the localization of parasite antigens using antibodies. The objective of this study was to identify the binding site of the polyclonal antibodies to the SGE and the salivary secretion using Perioxidase immunohistochemical staining.

Materials And Methods

Teneral horn flies (24-48 hrs. old) were immobilized by chilling at -20°C for about 10 minutes then fixed in 10%

phosphate neutral buffered formalin for about an hour. There were then transferred into 70% ethanol for an hour, embedded in paraffin and the head and thorax longitudinally sectioned at 6 um, and mounted on sialinized slides. The paraffin was removed from the sections by a 10 minute incubation in xylene and a subsequent wash in a graded ethanol series of decreasing concentration followed by a 10 min incubation in phosphate buffered saline (PBS). A modification of the ABC technique was performed (Haines and Chelack, 1991). Endogenous peroxidase was expended in deparaffinized sections by incubation in 3% H_2O_2 for 10 minutes at room temperature. Specimens were then incubated in 0.125% trypsin and 0.1% $CaCl_2$ for 20 minutes at 37° C. Nonspecific binding of biotinylated goat anti-mouse IgG and goat anti-rabbit IgG was blocked by incubation of the slides for the two different procedures either in normal mouse serum (NMS) or normal rabbit serum (NRS) (1:50 dilution in PBS) for 30 minutes at room temperature. All slides were then incubated for 1 hour at 37° C with different dilutions of primary mouse or rabbit polyclonal antibody preparation appropriately diluted in PBS supplemented with 1 % bovine serum albumin (PBS-BSA). After incubation, the slides were washed 3 times (10 min each) in PBS. The slides were then incubated for 1 hour at 37° C with biotinylated goat anti-mouse IgG or goat anti-rabbit IgG specific secondary antibody diluted to the manufacturers specifications (Sigma

Chemical, St. Louis MO) in PBS-BSA. Antigen detection was completed using the avidin-biotin-peroxidase complex method and kit (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). The slides were washed 3 times in PBS before being incubated for 1 hr at room temperature with a horseradish peroxidase-avidin-biotin conjugate (ABC reagent). All slides were then washed 3 times in PBS and subsequently incubated with 0.03% diaminobenzidine (with 1 drop of 3% H_2O_2) for approximately 5 minutes. Color development was visually monitored and the reaction stopped by washing the slides in several rinses of deionized water when satisfactory staining was achieved. All slides were counter-stained with Harris hematoxylin and cover slip added following standard laboratory procedures. Photographs of the sections were taken with a Zeiss II RS photomicroscope.

Results

Ultrathin sections of the head and thorax of the horn fly showed the ommatidia (O) making up the compound eye, The brain (supra-esophageal ganglion) (g) and epithelial cells of the salivary gland. Immunohistochemical staining with the sera from the exposed mouse and normal mouse showed non-specific binding. The antigenic material from the salivary gland could not be detected by the staining technique. There was intense chocolate brown staining of the epithelial cells of the salivary gland cells by the

peroxidase-conjugated rabbit anti-SGE serum. There was no immunostaining with the NRS. The ultrathin section reacted with rabbit antisera showed a distinct difference as compared to that of reacted with the normal rabbit serum. The antibody binding was very specific and did not react with other tissues in the ultrathin section. Rabbit anti-serum dilution of 1/200 was found to offer the best staining.

Discussion

The salivary glands are major targets of host immunoglobulin in ticks (Wikel et al., 1992). The saliva of hematophagous arthropods contain many biologically active molecules which enables them to obtain blood meals while avoiding host defenses of hemostasis, inflammation and immunity (Ribeiro, 1987). Therefore disrupting the salivary secretion could potentially reduce the fitness of the arthropod.

Immunohistochemical staining relies on the ability of the primary antibody to bind to the antigen of interest in the tissue specimen. The inability to detect specific staining with the primary mouse serum as discussed previously may be because a critical antigen mass was not

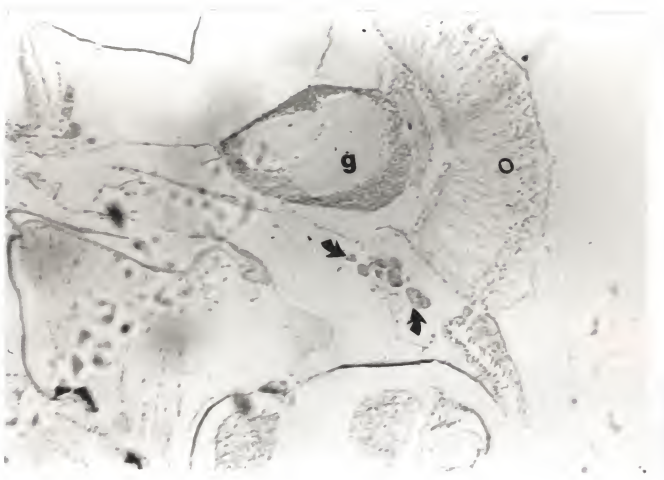

Fig 4.1. Light micrographs of longitudinal sections through the through head and thorax of horn fly immunohistochemically stained with normal mouse serum (NMS). No specific staining was noted or demonstrated. (O) Ommatidia, (g) brain and arrows denotes salivary gland epithelial cells. Scale bar = 0.5 mm.

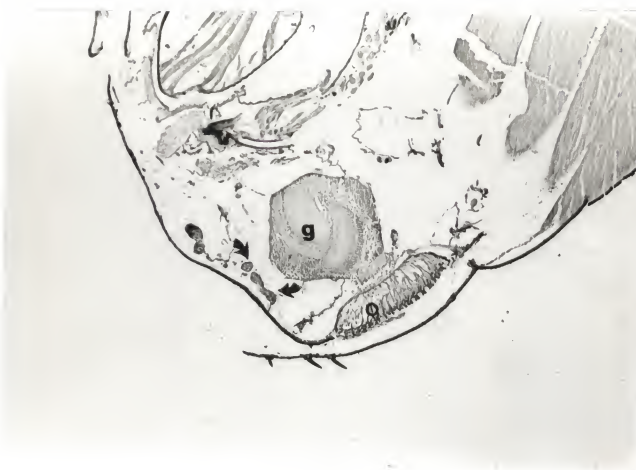

Figure 4.2. Light micrographs of longitudinal section through the head and thorax of the horn fly immunohistochemically stained with mouse anti-horn fly salivary secretion serum. No specific staining was noted or demonstrated. (o) ommatidia, (g) brain and arrow denoted the salivary gland epithelial cells. Scale bar = 0.5 um.

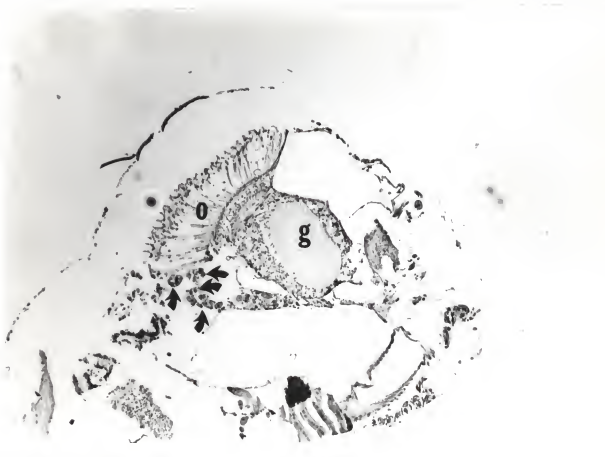

Figure 4.3. Light micrograph of longitudinal section through the head and thorax of horn fly immunohistochemically stained with normal rabbit serum (NRS). No specific staining was noted or observed. (O) ommatidia, (g) brain and arrows denote salivary gland epithelial cells. Scale bar = 0.5 mm

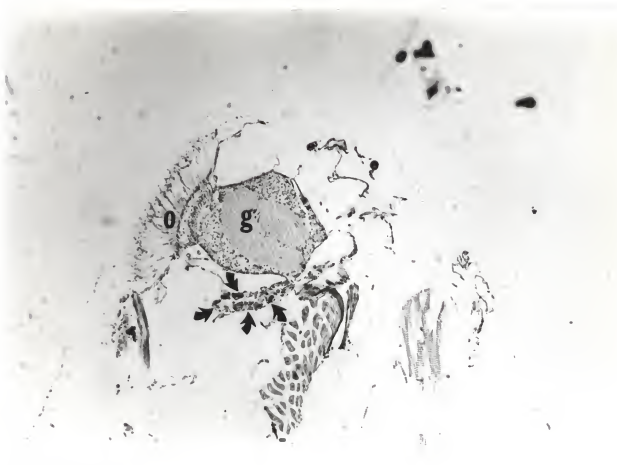

Figure 4.4. Light micrograph of longitudinal section through the head and thorax of the horn fly immunohistochemically stained with immunized rabbit serum. This shows specific immunohistochemical staining of salivary epithelium. Membrane and cytoplasmic staining was noted or demonstrated. (O) ommatidia, (g) brain and the arrows denote salivary gland epithelial cells. Scale bar = 0.5 mm.

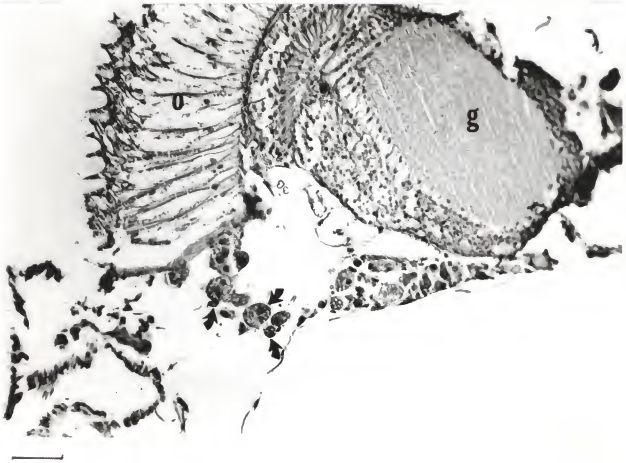

Figure 4.5. Higher magnification of specific immunohistochemical staining of salivary epithelium. (O) ommatidia, (g) brain and arrows denotes salivary gland epithelial cells. Scale bar = 0.1 mm.

secreted by the flies so little mouse antibody response was elicited and this probably was too low to be visualized by immunohistochemical staining. It may also be due to the low level of salivary secretion antigen expression in the host. The use of 10% neutral buffered formalin for fixation of the tissues in the histological sections could have reduced antigenicity of the tissues (Haines and Chelack, 1991). The loss of antigenicity varies with the type and duration of fixation and with the epitope detected by the antibodies. One of the characteristics of an antibody for immunohistochemical staining is that it must combine strongly with its antigen so as not to be washed off the tissue during the staining process (Polak and Van Noorden, 1984). Endogenous perioxidase activity in the salivary gland cells may also have caused the non-specific staining. These endogenous enzymes in tissues cause enzyme substrate deposition in the absence of specific antibody binding (Haines and Chelack, 1991). This deposition was controlled by inactivation of endogenous enzymes in the tissues prior to immunostaining. It is possible that some enzymes were still produced. The specific staining of the salivary gland epithelial cells by the primary immunized rabbit serum and the non-staining of the NRS shows that the polyclonal antibodies produced in the rabbit is specific to the salivary gland cells. No staining was observed on other tissues present in the ultrathin sections. The rabbits

developed antibodies with specificity for salivary glands. This shows that the salivary gland could provide a target of opportunity for host antibodies. The salivary glands are major targets for host antibodies in ticks (Wikel et al., 1992). Therefore it could be possible to manipulate the antibody in order to cause pathology in the horn fly.

Antigen tissue detection by the antibodies of the host animals provide good results but does not tell us about the effects on the horn fly. Allingham et al. (1992) detected intact host immunoglobulin G in the hemolymph of blood fed buffalo flies. The immunoglobulin was able to bind antigen. Antibodies transverse the midgut wall intact in both ixodid and argasid ticks, enter the hemocoel, and bind to various organs (Ackerman et al., 1981; Brossard et al., 1981; Brossard and Rais, 1983). Immunity in ticks induced by vaccination acts partly by causing direct damage to the parasite gut (Agbede and Kemp, 1986; Kemp et al., 1989). The damage causes leakage of blood components into the hemocoel and lead to death (Kemp et al., 1989). Reduction in mean engorged weight was the most consistent parameter for assessing vaccination-induced protective immunity in animals (Rechav and Dauth, 1987). One important aspect of host immune response that needs intense study is the ability of some arthropods especially ticks to evade the host immune system (Fielden et al., 1992). Detailed studies are needed to elucidate the effects of host antibodies on horn flies.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The purpose of this study was to immunologically characterize the major antigens in the salivary glands of the horn fly that can be attacked by host antibody and also to use a multiport olfactometer to determine the attractancy and repellency of hexane washes of individual cows in an attempt to characterize acquired host response as well as innate response to flies. The results show that the mouse and the rabbit mount strong immune responses to salivary secretion and salivary gland extracts, Immunized rabbit and infested mice both produced sera that was reactive with 27 KDa protein. The response of the immunized rabbit was also greater than that of the infested mice. This may mean that immunization could greatly increase antibody levels and be more effective than naturally induced infestation of hosts. This phenomenon of host immunological reactivity to arthropod feeding has been observed with many arthropod species but it has been intensely studied with ticks (Trager, 1939; Allen and Nelson, 1982; Shapiro et al., 1986; Willadsen, 1977). This results obtained in this study shows that the mice and the rabbit can be used as laboratory models to study horn fly host immune response, since it is

very cumbersome and expensive to use the horn fly's natural host, the cow. The use of the two types of antibody production (immunization and infestation) techniques showed a difference in the response of hosts immunized and those naturally exposed to horn fly feeding. The identification of the 27 KDa protein band as secretory protein may mean that the horn fly salivary secretion contains very few antigens or that it contains many but that the others evade the host immune response. Horn fly is an obligate ectoparasite, and it must not stimulate the host immune system greatly since this can result in unfavorable conditions for feeding.

Olfactometer studies confirmed field observation of attractancy and repellency of extracts of animals and also of chemicals supplied by IFF. Natural repellents produced by animals were also compared to plant extracts.

Despite encouraging progress, efforts to produce anti-arthropod vaccine show little immediate prospect of replacing vector control as the most effective strategy vector control. It has been observed that though antibodies are produced against arthropod antigens immunity to the arthropod has not been achieved (Allingham et al., 1992; Kerlin and Allingham, 1992; Kerlin and Hughes, 1992; Minnifield et al., 1993). Use of immune system for effect control comes with its own drawbacks and dangers. It is known that although the immune system is capable of

protecting it also has the capability to be harmful when things go wrong.

Production and use of vaccine for control must be preceded by in depth analysis and these include the type of effector cell involved in resistance, the developmental stage of the parasite, and associated antigen(s) that are recognized, and the form in which these exogenous proteins are presented to the immune system.

It is also possible that a changing array of salivary proteins are injected during feeding thus making it difficult to develop a unique antigen.

The above study represents the fundamental questions that need be answered before any further studies can be initiated. To further this work the proteins elucidating the immune response need to be purified. Once purified it can be used to isolate RNA then proceed to make cDNA which can be inserted in a bacteria to produce the antigen *in vitro* for vaccine production.

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BIOGRAPHICAL SKETCH

James Spencer Okine was born on October 16, 1958, in Accra, Ghana. Following graduation from Aggrey Memorial Zion Secondary School in 1978, during which he obtained his GCE Ordinary and Advanced level certificates, he entered the University of Ghana graduating in 1982 with a BSc in zoology\botany. He joined the Animal Research Institute as an Assistant Research officer in November 1982 to work on the use of an integrated control approach to control the tsetse fly. He received advanced training on the use of radioisotopes and radiation in entomology in Austria 1985 and the United States in 1986. In spring of 1989 he enrolled as a graduate student at the University of Florida. He graduated in spring 1991 with an MS. He was readmitted to the same department to pursue a Ph.D. program.

He is married to Grace and has three children Victor Ayittey, Earl Ayiquaye and Marilyn Okailey Okine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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